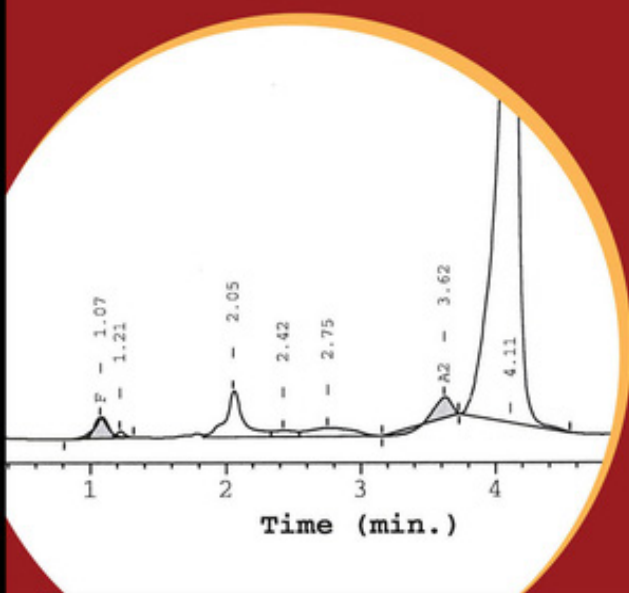


Fourth Edition

Haemoglobinopathy Diagnosis

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WILEY Blackwell

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Preface

This book is dedicated to the past and present scientific staff of the haematology departments of Princess Alexandra Hospital, Brisbane, Australia and St Mary's Hospital, Paddington, London (subsequently Imperial College Healthcare NHS Trust). It was the former group who first awakened Barbara Bain's interest in this field. They also suggested to her that there was a need for a practical book on the laboratory diagnosis of haemoglobinopathies and that she might be the person to write it. The second group, and in particular Lorry Phelan, for over four decades shared her pleasure in solving

diagnostic problems and, at the same time, providing an accurate, clinically relevant diagnostic service; subsequent staff members did the same. It is with much pleasure that we dedicate this new edition to these colleagues and friends.

We should also like to acknowledge many other colleagues throughout the world who have helped in diverse ways, including those who have contributed images. They are individually acknowledged in the figure legends.

Barbara J. Bain and David C. Rees
London, 2024

Abbreviations and glossary

- α** the Greek letter alpha
- α chain** the α globin chain, which is required for synthesis of haemoglobins A, F and A₂ and also the embryonic haemoglobin, Gower 2
- α gene** one of a pair of genes on chromosome 16, *HBA1* and *HBA2*, that encode α globin
- α thalassaemia** a group of thalassaemias characterised by absent or reduced α globin chain synthesis, usually resulting from deletion of one or more of the α globin genes; less often it results from altered structure of an α gene or mutation of the locus control gene, *LCRA*, or genes encoding *trans*-acting factors
- α⁰ thalassaemia** a thalassaemic condition in which there is no α globin chain translation from one or both copies of chromosome 16
- α⁺ thalassaemia** a thalassaemic condition in which there is reduced but not absent translation of α globin chain from one or both copies of chromosome 16
- β** the Greek letter beta
- β chain** the β globin chain, which forms part of haemoglobin A and haemoglobin Portland 2 and is the only globin chain in the abnormal haemoglobin, haemoglobin H
- β gene** the gene on chromosome 11, *HBB*, that encodes β globin
- β thalassaemia** a thalassaemia characterised by reduced β globin synthesis, usually caused by mutation of a β globin gene; less often it results from gene deletion or from deletion or mutation of the locus control region, *LCRB*
- β thalassaemia intermedia** a β thalassaemia with significant clinicopathological abnormalities but not dependent on transfusion for survival; also known as non-transfusion-dependent thalassaemia
- β thalassaemia major** a β thalassaemia with significant clinicopathological abnormalities, requiring transfusion to sustain life
- γ** the Greek letter gamma
- γ chain** the γ globin chain which forms part of fetal haemoglobin (haemoglobin F) and the embryonic haemoglobin, haemoglobin Portland 1, and is the only globin chain in the abnormal variant, haemoglobin Bart's
- γ gene** one of a pair of very similar genes on chromosome 11, *HBG1* and *HBG2*, encoding γ globin chain
- γ thalassaemia** a thalassaemic condition resulting from reduced synthesis of γ globin chain
- δ** the Greek letter delta
- δ chain** a β-like globin chain, which forms part of haemoglobin A₂
- δ gene** a gene of the β cluster on chromosome 11, *HBD*, that encodes δ globin
- δ thalassaemia** reduced or absent synthesis of δ globin and therefore of haemoglobin A₂
- ε** the Greek letter epsilon
- ε chain** the ε globin chain, which is synthesised during early embryonic life and forms part of haemoglobins Gower 1 and Gower 2
- ε gene** a gene of the α globin cluster on chromosome 16, *HBE1*, that encodes ε globin chain
- ψ** the Greek letter psi, used to indicate a pseudogene
- ζ** the Greek letter zeta
- ζ chain** the ζ globin chain that is synthesised in intrauterine life and that forms part of haemoglobins Gower 1, Portland 1 and Portland 2
- ζ gene** a gene of the α globin gene cluster on chromosome 16, *HBZ*, that encodes ζ globin chain
- 2,3 DPG** 2,3-diphosphoglycerate; a small molecule that interacts with haemoglobin, decreasing its oxygen affinity
- 3'** the end of a gene where transcription ceases
- 5'** the end of a gene where transcription starts
- acquired** a condition that is not present at birth or is not inherited

affinity the avidity of haemoglobin for oxygen

AHSP α haemoglobin stabilising protein (AHSP)

AIDS acquired immune deficiency syndrome

ala δ -aminolaevulinic acid, the first compound formed during the process of haem synthesis

AML acute myeloid leukaemia

ARMS amplification refractory mutation system, a PCR technique used, for example, for the detection of mutations causing β thalassaemia; it employs two primer sets, one amplifying normal sequences and one abnormal sequences

balanced polymorphism the stable persistence of two or more alleles of gene in a significant proportion of a population; a potentially deleterious allele may show balanced polymorphism if the heterozygous state conveys an advantage

base a ring-shaped organic molecule containing nitrogen, which is a constituent of DNA and RNA; DNA contains four bases: adenine, guanine, cytosine and thymine; RNA contains four bases: adenine, guanine, cytosine and uracil

Bohr effect the effect of pH on oxygen affinity; the alkaline Bohr effect is the reduction of oxygen affinity of haemoglobin as pH falls from above to below the physiological pH; there is also an acid Bohr effect which is a rise of oxygen affinity as the pH falls further, at a pH level that is incompatible with life

bp base pair, the pairing of specific bases, e.g. adenine with thymine, in the complementary strands of the DNA double helix

CAP 7-methyl guanosine cap, added to RNA molecule during processing

capillary electrophoresis electrophoresis within a capillary tube

carbonic anhydrase a red cell enzyme that is the second most abundant red cell protein after haemoglobin; it may be apparent on haemoglobin electrophoretic strips if a protein rather than a haem stain is used

carboxyhaemoglobin haemoglobin that has been chemically altered by combination with carbon monoxide

CE-HPLC cation-exchange high performance liquid chromatography; *see* HPLC

chromatography a method of separating proteins from each other by means of physical

characteristics, such as molecular weight, charge or hydrophobicity, or by means of differing affinity for lectins, antibodies or other proteins; in column chromatography the proteins move through an absorbent column and emerge after different periods of time

cis on the same chromosome (see also *trans*)

cis-acting a DNA sequence that affects the expression of a gene on the same chromosome but not on the homologous chromosome (see also *trans-acting*)

CNV copy number variant

CO carbon monoxide, the molecule composed of one carbon atom and one oxygen atom, formed by combustion of hydrocarbons

CO₂ carbon dioxide, the molecule composed of one atom of carbon combined with two atoms of oxygen

codon a triplet of nucleotides that encodes a specific amino acid or serves as a termination signal; there are 61 codons encoding 20 amino acids and three codons that act as termination or STOP codons

congenital present at birth, often but not necessarily inherited

cooperativity the interaction between the four globin monomers that makes possible the Bohr effect and the sigmoid shape of the oxygen dissociation curve

COVID-19 corona virus disease 2019

CT computed tomography

CV coefficient of variation

DCIP test a screening test for haemoglobin E using dichlorophenolindophenol

deletion loss of part of a chromosome, which may include all or part of a globin gene

deoxyhaemoglobin haemoglobin that is not combined with O₂

DGGE denaturing gradient gel electrophoresis, a molecular genetic technique for locating a mutation prior to precise analysis

DNA deoxyribonucleic acid, the major constituent of the nucleus of a cell; a polynucleotide strand that is able to replicate and that codes for the majority of proteins synthesised by the cell; the DNA molecule is a double helix of two complementary intertwined polynucleotides

EDTA ethylene diamine tetra-acetic acid

eIF2 erythroid initiation factor 2

- EKLF** erythroid Krüppel-like factor
- electrophoresis** separation of charged suspended particles such as proteins by application to a membrane or gel or within a capillary followed by exposure to a charge gradient, e.g. haemoglobin electrophoresis
- ELISA** enzyme-linked immunosorbent assay
- elution** removal of an absorbed substance from a chromatography column or membrane
- enhancer** a DNA sequence that influences the promoter of a nearby gene to increase transcription; an enhancer acts on a gene in *cis* and may be sited upstream, downstream or within a gene
- exon** a part of a gene that is represented in mature messenger RNA; most genes are composed of exons and non-translated introns
- FAB classification** French–American–British classification (of acute leukaemia)
- FBC** full blood count
- Fe** iron
- Fe⁺⁺, Fe²⁺** ferrous or bivalent iron
- Fe⁺⁺⁺, Fe³⁺** ferric or trivalent iron
- fetal haemoglobin** *see* haemoglobin F
- G6PD** glucose-6-phosphate dehydrogenase
- GAP-PCR** a PCR technique in which there is amplification across a 'gap' created by a deletion
- GATA1** an erythroid-specific transcription factor
- GDP** guanosine diphosphate
- gene** the segment of DNA that is involved in producing a polypeptide chain; it includes regions preceding and following the coding region (5' and 3' untranslated regions) as well as intervening sequences (introns) between individual coding segments (exons); genes mediate inheritance; they are located on nuclear chromosomes or, for a minority of genes, in a mitochondrion
- genetic code** the relationship between a triplet of bases, called a codon, and the amino acid that it encodes
- genotype** the genetic constitution of an individual (c.f. phenotype)
- globin** the protein part of the haemoglobin molecule, usually composed of two pairs of non-identical chains, e.g. two α chains and two β chains
- GTP guanosine triphosphate
- H⁺** a proton
- haem** a porphyrin structure that contains iron and that forms part of the haemoglobin molecule
- haemoglobin** a complex molecule composed of four globin chains, each one enclosing a haem group
- haemoglobin A** the major haemoglobin component present in most adults, having two α and two β chains
- haemoglobin A_{1c}** glycosylated haemoglobin A
- haemoglobin A₂** a minor haemoglobin component present in almost all adults and, as an even lower proportion of total haemoglobin, in neonates and infants, having two α chains and two δ chains
- haemoglobin A₂'** a haemoglobin A₂ variant, also known as haemoglobin B₂
- haemoglobin Bart's** an abnormal haemoglobin with four γ chains and no α chains, present as the major haemoglobin component in haemoglobin Bart's hydrops fetalis and as a minor component in neonates with haemoglobin H disease or alpha thalassaemia trait
- haemoglobin Bart's hydrops fetalis** a fatal condition of a fetus or neonate with no α genes and consequently no production of haemoglobins A, A₂, F or Gower 2
- haemoglobin C** a variant haemoglobin with an amino acid substitution in the β chain, mainly found in those of African ancestry
- haemoglobin Constant Spring** a variant haemoglobin with a structurally abnormal α chain that is synthesised at a reduced rate, leading to α thalassaemia
- haemoglobin D** the designation of a group of haemoglobin variants, some α chain variants and some β chain variants, that have the same mobility as haemoglobin S on electrophoresis at alkaline pH
- haemoglobin dissociation curve** a plot of percentage saturation of haemoglobin against partial pressure of oxygen
- haemoglobin E** a variant haemoglobin with an amino acid substitution in the β chain, mainly found in South-East Asia and parts of the Indian subcontinent
- haemoglobin F** fetal haemoglobin, the major haemoglobin of the fetus and neonate, having

- two α chains and two γ chains; also present as a very minor component in most adults and as a larger proportion in a minority
- haemoglobin G** the designation of a group of haemoglobin variants, some α chain variants and some β chain variants, that have the same mobility as haemoglobin S on electrophoresis at alkaline pH
- haemoglobin Gower 1** an embryonic haemoglobin, having two ζ chains and two ϵ chains
- haemoglobin Gower 2** an embryonic haemoglobin, having two α chains and two ϵ chains
- haemoglobin H** a variant haemoglobin with four β chains and no α chains, present in haemoglobin H disease and, in small quantities, in α thalassaemia trait
- haemoglobin H disease** a haemoglobinopathy caused by marked underproduction of α chains, often consequent on deletion of three of the four α genes
- haemoglobin I** a group of variant haemoglobins that move more rapidly than haemoglobin A on electrophoresis at alkaline pH
- haemoglobin J** a group of variant haemoglobins that move more rapidly than haemoglobin A but more slowly than haemoglobin I on electrophoresis at alkaline pH
- haemoglobin K** a group of variant haemoglobins moving between A and J on electrophoresis at alkaline pH
- haemoglobin Lepore** a number of variant haemoglobins resulting from the fusion of part of a δ globin gene with part of a β globin gene, giving a $\delta\beta$ fusion gene and a fusion protein that combines with α globin to form haemoglobin Lepore
- haemoglobin M** a variant haemoglobin that oxidises readily to methaemoglobin
- haemoglobin N** a group of variant haemoglobins moving between J and I on electrophoresis at alkaline pH
- haemoglobin O-Arab** a β chain variant haemoglobin moving near C at alkaline pH and near S at acid pH
- haemoglobinopathy** an inherited disorder resulting from synthesis of a structurally abnormal haemoglobin; the term can also be used to encompass, in addition, the thalassaemias in which there is a reduced rate of synthesis of one of the globin chains
- haemoglobin Portland 1** an embryonic haemoglobin, having two ζ chains and two γ chains
- haemoglobin Portland 2** abnormal embryonic haemoglobin, having two ζ chains and two β chains, present in some severe thalassaemia syndromes
- haemoglobin S** sickle cell haemoglobin, a variant haemoglobin with a tendency to polymerise at low oxygen tension, causing erythrocytes to deform into the shape of a sickle
- Hb** haemoglobin concentration
- Hct** haematocrit
- HDAC1** histone deacetylase 1
- HDW** haemoglobin distribution width
- heteroduplex analysis** a molecular genetic technique for locating a mutation prior to precise analysis
- heterozygosity** the state of having two different alleles of a specified autosomal gene or, in a female, two different alleles of an X chromosomal gene
- heterozygous** having two different alleles of a specified autosomal or X chromosome gene
- HIV** human immunodeficiency virus
- homologue** an equivalent or similar structure; the $\alpha 1$ and $\alpha 2$ genes are homologues, as are the two copies of a chromosome
- homologous** being equivalent or similar to another
- homology** the presence of structural similarity, implying a common remote origin; the δ and β genes show partial homology
- homozygosity** the state of having two identical alleles of a specified autosomal or X chromosome gene
- homozygous** having two identical alleles of a specified autosomal gene or, in a female, two identical alleles of an X chromosome gene)
- HPFH** hereditary persistence of fetal haemoglobin
- HPLC** high performance liquid chromatography, a method of separating proteins, such as haemoglobin variants, from each other on the basis of characteristics such as size, hydrophobicity and ionic strength; a solution of proteins is eluted from a specially designed column by exposure to various buffers, different proteins emerging after varying periods of time

- HRI** haem-regulated inhibitor
- HS1, HS2, HS3, HS4** hypersensitive sites 1, 2, 3, and 4, upstream of the β globin gene cluster
- HS -40** an upstream enhancer of α globin gene transcription, part of *LCRA*
- HVR** hypervariable region
- ICSH** International Council for Standardization in Haematology
- IEF** isoelectric focusing, the separation of proteins in an electric field as they move through a pH gradient to their isoelectric points
- inherited** a characteristic that is transmitted from a parent, by means of genes that form part of nuclear or mitochondrial DNA
- initiation** (i) the process by which RNA transcription from a gene commences; (ii) the process by which protein translation from mRNA commences
- initiation codon** the three-nucleotide codon (ATG) at the 5' end of a gene that is essential to permit initiation of transcription of a gene, i.e. initiation of polypeptide synthesis
- insertion** the insertion of a DNA sequence, e.g. from one chromosome into another
- intervening sequence** an intron
- intron** a sequence of DNA in a gene that is not represented in processed messenger RNA or in the protein product
- inversion** the reversal of the normal position of a DNA sequence on a chromosome
- isoelectric point** the pH at which a protein has no net charge
- IVS** intervening sequence, intron
- kb** kilobase, a unit for measuring the length of DNA; one kilobase is 1000 nucleotide base pairs
- kD** kilodalton, a unit for measuring molecular weight; one kilodalton is 1000 daltons
- KLF1** Krüppel-like factor 1
- LCR** locus control region, a DNA sequence upstream of genes of the α or β globin cluster that enhances transcription of the genes of the cluster, *LCRA* and *LCRB* control the α and β gene clusters respectively
- LCRA** locus control region alpha
- LCRB** locus control region beta
- LDH** lactate dehydrogenase
- MCH** mean cell haemoglobin
- MCHC** mean cell haemoglobin concentration
- MCV** mean cell volume
- MDS** myelodysplastic syndrome
- methaemoglobin** oxidised haemoglobin, which does not function in oxygen transport
- MGG** May–Grünwald–Giemsa (stain)
- mis-sense mutation** a mutation that leads to the encoding of a different amino acid
- MPLA** multiple ligation-dependent probe amplification
- MRI** magnetic resonance imaging
- mRNA** messenger RNA, ribonucleic acid that is transcribed in the nucleus, on a DNA template, and moves to the cytoplasm, becoming attached to ribosomes and serving as a template for synthesis of proteins
- MS** mass spectrometry, electrospray ionisation mass spectrometry, a method for determining the mass and the charge of a molecule
- NO** nitric oxide
- nonsense mutation** a mutation that leads to no amino acid being encoded that therefore functions as a STOP or termination codon, leading to synthesis of a truncated polypeptide chain
- non-transfusion-dependent thalassaemia** a thalassaemia with significant clinicopathological abnormalities but not dependent on transfusion for survival, also known as β thalassaemia intermedia
- NRBC** nucleated red blood cell/cells
- NTDT** non-transfusion-dependent thalassaemia
- O₂** oxygen
- ORF** open reading frame
- oxyhaemoglobin** haemoglobin combined with O₂
- P₅₀ PO₂** at which haemoglobin is half saturated
- PaO₂** partial pressure of oxygen in arterial blood
- partial pressure of oxygen** that part of the total blood gas pressure exerted by oxygen
- PAS** periodic acid–Schiff (stain)
- PCR** polymerase chain reaction, a method of making multiple copies of a DNA sequence
- PCV** packed cell volume, haematocrit
- phenocopy** a condition that simulates an inherited condition; a phenocopy may be acquired or may be a genetic characteristic that simulates another
- phenotype** the characteristics of an individual, which may be determined by the genotype or may be an acquired characteristic (c.f. genotype)

PO₂ partial pressure of oxygen

polymorphism the occurrence of a variant form of a gene in a significant proportion (at least 1%) of a population

promoter a sequence of DNA at the 5' end of a gene that is essential for initiation of transcription

pseudogene a non-functioning homologue of a gene

purine one of the two types of nitrogenous base found in nucleic acids; purines have a double ring structure (see also *pyrimidine*)

pyrimidine one of the two types of nitrogenous base found in nucleic acids; pyrimidines have a single ring structure (see also *purine*)

RBC red blood cell (count)

RDW red cell distribution width, a measure of anisocytosis

restriction endonuclease an enzyme that recognises specific sequences in a DNA molecule and cleaves the molecule in or very near the recognition site

restriction fragment a fragment of DNA produced by cleavage by a restriction endonuclease

RFLP restriction fragment length polymorphism, variation between homologous chromosomes with regard to the length of DNA fragments produced by application of a specific restriction endonuclease; can be used for the demonstration of heterozygosity or for demonstration of a specific gene that removes or creates a specific cleavage site

ribosome a cytoplasmic structure on which proteins are translated from messenger RNA; ribosomes may be free within the cytosol or form part of the rough endoplasmic reticulum

RNA ribonucleic acid, a polynucleotide in which the nitrogenous bases are adenine, guanine, cytosine and uracil and the sugar is ribose; RNA is produced in the nucleus and in mitochondria from DNA templates

rRNA ribosomal RNA, RNA that, together with protein, constitutes the ribosomes

SARS-CoV-2 severe acute respiratory syndrome-corona virus-2

sickle cell an erythrocyte that has become sickle or crescent shaped as a result of polymerisation of haemoglobin S

sickle cell anaemia the disease resulting from homozygosity for haemoglobin S

sickle cell disease a group of diseases including sickle cell anaemia and various compound heterozygous states in which clinicopathological effects occur as a result of sickle cell formation (preferred definition but sometimes used as a synonym for sickle cell anaemia)

sickle cell trait heterozygosity for the β^S gene that encodes the β chain of haemoglobin S

SNP single nucleotide polymorphism

SOP standard operating procedure

splicing the process by which RNA sequences corresponding to introns in the gene are removed during processing of RNA

SSP stage selector protein

sulphaemoglobin haemoglobin that has been irreversibly oxidised and chemically altered by drugs or chemicals with incorporation of a sulphur atom into the haemoglobin molecule

thalassaemia a disorder, almost always inherited, in which one or more of the globin chains incorporated into a haemoglobin molecule or molecules is synthesised at a reduced rate

thalassaemia intermedia a genetically heterogeneous thalassaemic condition that is moderately severe but nevertheless does not require regular blood transfusions to sustain life; usually refers to β thalassaemia intermedia

thalassaemia major thalassaemia that is incompatible with more than a short survival in the absence of blood transfusion; usually refers to β thalassaemia major

thalassaemia minor an asymptomatic thalassaemic condition, attributable to β thalassaemia heterozygosity or to deletion of one or two of the four α genes; usually referred to as thalassaemia trait

trait a term applied to heterozygosity for an inherited characteristic; in the case of disorders of globin genes, the term would not be used if heterozygosity were associated with a significant phenotypic abnormality; rather it is used when homozygosity or compound heterozygosity produces a clinically significant abnormality but simple heterozygosity does not

trans having an influence on a DNA sequence on another chromosome (see also *cis*)

trans-acting a DNA sequence that affects the expression of a gene on another chromosome (see also *cis-acting*)

transcript an RNA molecule, corresponding to one gene, transcribed from nuclear DNA

transcription the synthesis of RNA on a DNA template

transcription factor a protein capable of enhancing transcription of one or more genes

translation the synthesis of protein from an mRNA template

tRNA transfer RNA, RNA molecules that bind to specific amino acids and transport them to ribosomes; there they bind to specific mRNA

sequences, leading to incorporation of amino acids into peptide chains in the sequence specified by the mRNA

unstable a term applied to a haemoglobin that is abnormally prone to post-translational structural alteration, which may include loss of the normal tertiary or quaternary structure

UTR untranslated region

variant a term applied to any haemoglobin other than haemoglobins A, A₂, F and the normal embryonal haemoglobins

WBC white blood cell (count)

yolk sac a membranous sac attached to an embryo, the initial site of formation of blood cells

1 Haemoglobin and the genetics of haemoglobin synthesis

Haemoglobins and their structure and function

The haemoglobin molecule contained within red blood cells is essential for human life, being the means by which oxygen is transported to the tissues. Other functions include the transport of carbon dioxide (CO_2) and a buffering action (reduction of the changes in pH that would otherwise be expected when an acid or an alkali enters or is generated in a red cell). A normal haemoglobin molecule has a molecular weight of 64–64.5 kDa and is composed of two dissimilar pairs of polypeptide chains, each of which encloses an iron-containing porphyrin designated haem (Fig. 1.1). Haem is essential for oxygen transport while globin serves to protect haem from oxidation, renders the molecule soluble and permits variation in oxygen affinity. The structure of the haemoglobin molecule produces an internal environment of hydrophobic radicals, which protects the iron of haem from water and thus from oxidation. External radicals are hydrophilic and thus render the haemoglobin molecule soluble. Both haem and globin are subject to modifications. The iron of haemoglobin is normally in the ferrous form (Fe^{2+}). Haem is able to combine reversibly with oxygen so that haemoglobin can function as an oxygen-transporting protein. Oxidation of iron to the ferric form (Fe^{3+}) is a less readily reversible reaction, converting haem to haematin and haemoglobin to methaemoglobin, a form of haemoglobin that cannot transport oxygen. Auto-oxidation of haemoglobin to methaemoglobin is a normal process. About 3% of haemoglobin undergoes this process each day with about 1%

(0.4–1% in one study) of haemoglobin being methaemoglobin [1, 2]. Methaemoglobin is converted back to haemoglobin mainly by the action of NADH-cytochrome b5-methaemoglobin reductase.

The haemoglobin molecule can also combine with CO_2 , haemoglobin being responsible for about 10% of its transport from the tissues to the lungs; transport is by reversible carbamation of the N-terminal groups of the α chains of haemoglobin. Because carbamated haemoglobin has a lower oxygen affinity than the non-carbamated form, binding of the CO_2 produced by the metabolic processes in tissues facilitates oxygen delivery to tissues. In addition, non-oxygenated haemoglobin can carry more CO_2 than oxygenated haemoglobin so that unloading of oxygen to the tissues facilitates the uptake and transport of CO_2 . Because of its buffering action (mopping up of protons, H^+), haemoglobin also contributes to keeping CO_2 in the soluble bicarbonate form and thus transportable. The reaction $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+$ is facilitated.

Haemoglobin also has a role in nitric oxide (NO) transport and metabolism. Haemoglobin is both a scavenger of nitric oxide and an active transporter. Nitric oxide is produced in endothelial cells and neutrophils by the action of nitric oxide synthases [2–5]. It has a very high affinity for oxyhaemoglobin so that blood levels are a balance between production and removal by binding to oxyhaemoglobin. Nitric oxide is a potent vasodilator, but this effect is limited by its binding to haemoglobin. The iron atom of a haem group of oxyhaemoglobin (preferentially the haem enclosed in the haem pocket of an

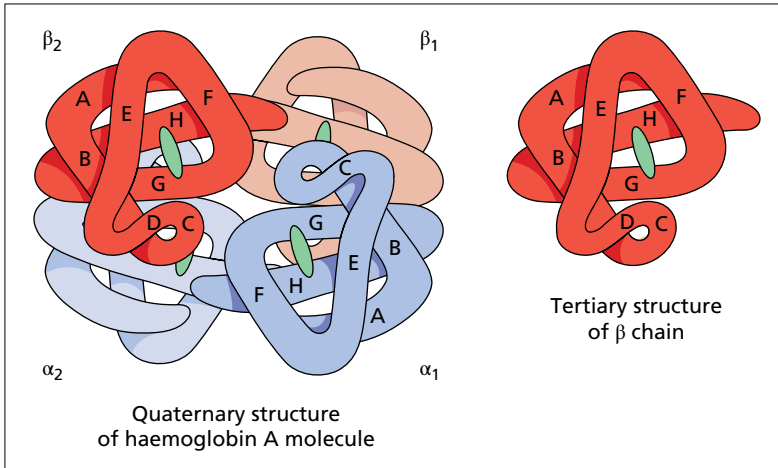


Fig. 1.1 Diagrammatic representation of the tertiary structure of a haemoglobin monomer (a β globin chain containing a haem group) and the quaternary structure of haemoglobin; upper case letters indicate homologous α helices.

α chain), binds nitric oxide. A haemoglobin molecule with nitric oxide bound to two haem groups strikingly favours the deoxy conformation so oxygen is readily released. Nitric oxide-haemoglobin is subsequently converted to methaemoglobin with release of nitric oxide and production of nitrate ions, which are excreted. Since deoxyhaemoglobin has a much lower affinity for nitric oxide, hypoxic conditions could leave more nitric oxide free and lead to vasodilation, which is of potential physiological benefit. In addition, deoxyhaemoglobin can convert nitrite to nitric oxide, again favouring vasodilation.

Nitric oxide also causes S-nitrosylation of a conserved cysteine residue (Cys⁹³, E15) of the β globin chain of oxyhaemoglobin to form S-nitrosohaemoglobin. This occurs in the lungs. In this circumstance, the bioactivity of nitric oxide may be retained with nitric oxide being delivered to low molecular weight thiol-containing molecules to reach target cells such as the smooth muscle of blood vessels. Oxygenation of haemoglobin favours S-nitrosylation. Conversely, deoxygenation favours release of nitric oxide. This may be an important physiological process with nitric oxide being released in peripheral tissues where it can facilitate arteriolar dilation. The oxy form of S-nitrosohaemoglobin is a vasoconstrictor whereas the deoxy form is a vasodilator. Lack of oxygen could thus again favour vasodilation.

In normal circumstances, the ability of haemoglobin to scavenge or destroy nitric oxide is reduced by the barrier to nitric oxide diffusion that is provided by the red cell membrane. However, in haemolytic anaemias with increased free plasma haemoglobin, binding and inactivation can be almost immediate, leading to impaired vascular responses to nitric oxide [5]; inactivation of nitric oxide by haemoglobin in the plasma may thus contribute to the pulmonary hypertension that can be a feature of sickle cell anaemia and also to the hypertension that has been observed with some haemoglobin-based blood substitutes.

Surprisingly, the α globin genes are expressed in endothelial cells with the α globin participating in nitric oxide scavenging [6]. Individuals with deletion of one or two α globin genes have enhanced nitric oxide-induced vasodilation [7].

As a result of the synthesis of different globin chains at different stages of life (Fig. 1.2) there is a difference in the type of haemoglobin present in red cells between adult life and the fetal and neonatal periods (Table 1.1, Fig. 1.3). In adults, 96–98% of haemoglobin is haemoglobin A (A = adult), which has two alpha (α) chains and two beta (β) chains. The name 'haemoglobin A' was given by Linus Pauling and colleagues in 1949 when they discovered that asymptomatic carriers of sickle cell disease had two different haemoglobins, which they designated haemoglobin A and haemoglobin S [8]. A minor

Fig. 1.2 Diagrammatic representation of the sites and rates of synthesis of different globin chains in the embryonic and fetal periods and during infancy.

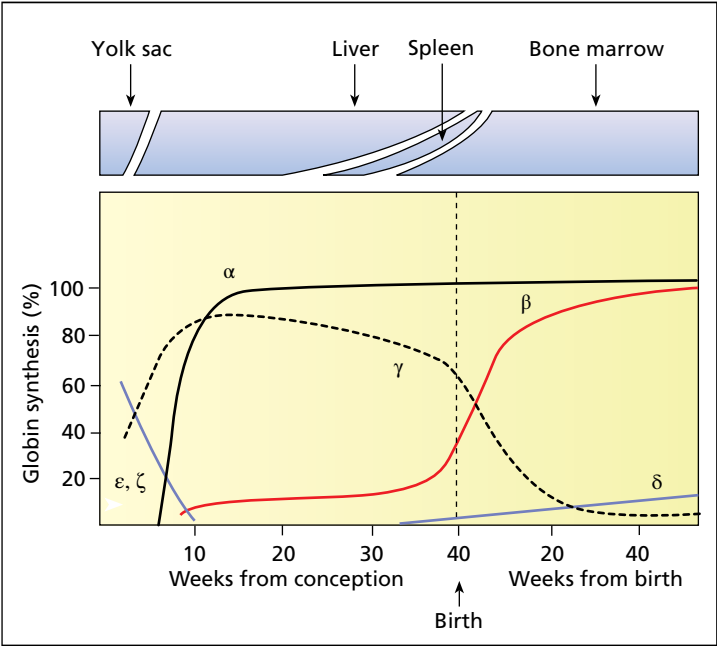


Table 1.1 Haemoglobins normally present during adult, fetal and embryonic periods of life.

Haemoglobin species	Globin chains	Period when normally present
A	$\alpha_2\beta_2^*$	Major haemoglobin in adult life
A ₂	$\alpha_2\delta_2$	Minor haemoglobin in adult life; even more minor in late fetal and neonatal life
F	$\alpha_2^G\gamma_2, \alpha_2^A\gamma_2$ or $\alpha_2^A\gamma^G\gamma$	Minor haemoglobin in adult life, major haemoglobin in fetal life with a declining percentage through the neonatal period
Gower 1	$\zeta_2\epsilon_2$	Significant haemoglobin during early intrauterine life
Gower 2	$\alpha_2\epsilon_2$	Significant haemoglobin during early intrauterine life
Portland or Portland 1†	$\zeta_2\gamma_2$	Significant haemoglobin during early intrauterine life

* Can also be designated $\alpha^A_2\beta^A_2$ to distinguish the globin chains of haemoglobin A from those of variant haemoglobins.
† Haemoglobin Portland 2 ($\zeta_2\beta_2$) has been observed in α thalassaemia syndromes but is unlikely to occur in significant amounts during normal development.

haemoglobin, haemoglobin A₂, has two α chains and two delta (δ) chains. Its existence was first reported in 1955 by Kunkel and Wallenius [9]; they noted its increased level in thalassaemia minor and that it was reduced or absent in neonates. A very minor haemoglobin in adults but the major haemoglobin during fetal life and the early neonatal period is haemoglobin F or

fetal haemoglobin, which has two α chains and two gamma (γ) chains. There are two species of haemoglobin F, designated $^G\gamma$ and $^A\gamma$, with glycine and alanine respectively at position 136 of the γ chain. In addition, the $^A\gamma$ chain shows polymorphism at position 75, which may be occupied by threonine rather than the more common isoleucine [10], a polymorphism that

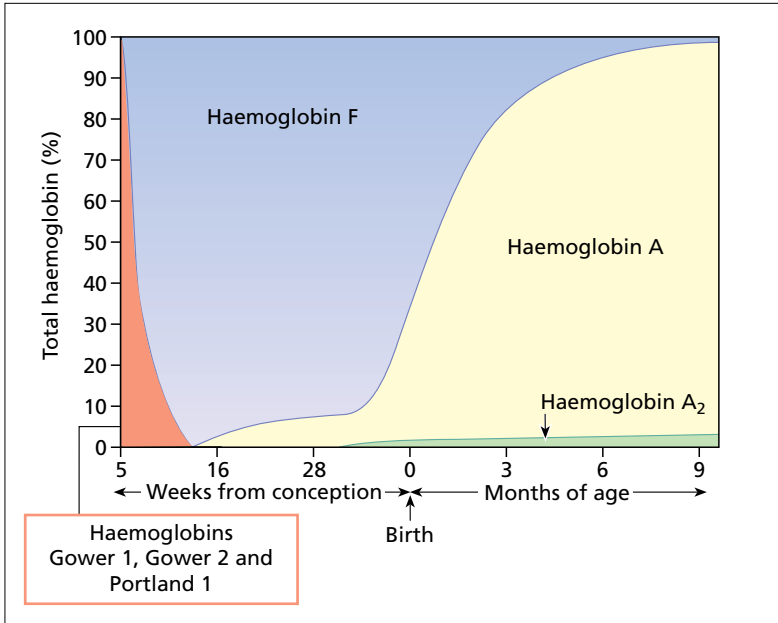


Fig. 1.3 Diagrammatic representation of the average percentages of various haemoglobins present in the embryonic and fetal periods and during infancy.

was previously referred to as haemoglobin F-Sardinia. In the early embryo, haemoglobin is synthesised in the yolk sac and specific embryonic haemoglobins are produced – Gower 1, Gower 2 and Portland (or Portland 1). They contain globin chains that are synthesised in significant amounts only during embryonic life, specifically zeta (ζ) and epsilon (ϵ) chains (see Table 1.1). Haemoglobins Gower 1 ($\zeta_2\epsilon_2$) and Gower 2 ($\alpha_2\epsilon_2$) were first described by Huehns and colleagues in 1961 [11], being named after Gower Street, in London, in which University College Hospital is situated. Portland 1 ($\zeta_2\gamma_2$) was described in 1967 and was so named because it was first identified in the University of Oregon in Portland, Oregon [12]. By five weeks of gestation, ζ and ϵ chains are already being synthesised in primitive erythroblasts in the yolk sac. From the sixth week onwards these same cells start to synthesise α , β and γ chains. Starting from about the 10th to the 12th week of gestation there is haemoglobin synthesis in the liver and the spleen with production of fetal and later adult haemoglobin. Production of the various embryonic, fetal and adult haemoglobins is synchronous in different sites. Later in intrauterine life, the bone marrow takes over as the main site of haemoglobin synthesis and

increasing amounts of haemoglobin A are produced. In adult life, bone marrow erythroblasts synthesise haemoglobin A and the minor haemoglobins.

The embryonic haemoglobins have a higher oxygen affinity than haemoglobin A, similar to that of haemoglobin F [13]. They differ from haemoglobins A and F in that they continue to bind oxygen strongly, even in acidotic conditions [13]. In the case of Gower 2, impaired binding to 2,3-diphosphoglycerate (2,3-DPG) is the basis of the increased oxygen affinity [14].

Formation of the haemoglobin A molecule starts with formation of an $\alpha\beta$ dimer. Normally α chains are produced in slight excess. Alpha haemoglobin stabilising protein (AHSP) acts as a molecular chaperone, facilitating formation of the dimer and preventing the precipitation of free α chains, which would lead to generation of reactive oxygen species with resultant damage to cells. Any free β chains are soluble.

Haemoglobin can undergo post-translational modifications (see also Chapter 6). Glycosylation occurs with formation of haemoglobins A_{1a-e} , but principally of haemoglobin A_{1c} . In normal individuals haemoglobin A_{1c} may constitute up to 4–6% of total haemoglobin but in patients with diabetes mellitus it can be much higher. It is also

increased in the acquired immune deficiency syndrome (AIDS) [15]. In individuals with a shortened red cell life span the percentage of haemoglobin A_{1c} is lower. Another minor fraction, formed on ageing, is haemoglobin A_{111} , in which glutathione is bound to the cysteine at $\beta 93$. Unmodified haemoglobin can be distinguished by use of the designation haemoglobin A_0 . In the fetus about 20% of haemoglobin F shows acetylation of the γ chain but this is not a major feature of other normal human globin chains [10]. Exposure to carbon monoxide, the product of incomplete combustion of hydrocarbons, leads to the formation of carboxyhaemoglobin. In normal individuals carboxyhaemoglobin comprises 0.2–0.8% of total haemoglobin but in heavy smokers it may be as much as 10–15%. Small amounts of sulphhaemoglobin (<0.5%) [1] and methaemoglobin are also formed in normal subjects. Methaemoglobin (see earlier) is usually less than 1% of total haemoglobin. Other post-translational modification of globin chains includes carbamylation, pyruvatisation and acetaldehyde adduct formation [16]. Glutathionylation is increased in diabetes mellitus [17] and by the administration of certain anti-epileptic drugs (phenobarbital and carbamazepine) [18]. Post-synthetic modification of a haemoglobin molecule can also occur as a consequence of a mutation in a globin gene; either the abnormal amino acid or an adjacent normal amino acid can undergo post-translational conversion to another amino acid (see later). In addition, some abnormal haemoglobins in which there is a mutation of N terminal amino acid are particularly prone to acetylation, which occurs co-translationally [19].

The structure of haemoglobin is highly complex and can be viewed at four levels.

1 The primary structure is the sequence of the amino acids in the polypeptide that constitutes the globin chain.

2 The secondary structure is the arrangement of the polypeptide globin chains into α helices (stabilised by hydrogen bonds) separated by non-helical turns. In the case of the β globin chain there are eight α helices, designated A to H, whereas the α globin chain lacks the D helix residues; 70–80% of the amino acid residues of haemoglobin form part of the helices.

3 The tertiary structure is the arrangement of the coiled globin chain into a three-dimensional structure that has a surface haem-containing pocket between the E and F helices; binding of haem between two specific histidine residues in the E and F helices respectively (Fig. 1.4) is essential for maintaining the secondary and the tertiary structure of haemoglobin.

4 The quaternary structure is the relationship between the four globin chains, which is not fixed. The strong $\alpha_1\beta_1$ and $\alpha_2\beta_2$ bonds (dimeric bonds) hold the molecule together in a stable form while the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ bonds (tetrameric bonds) both contribute to stability, albeit to a lesser extent than the dimeric bonds, and permit the chains to slide on each other and rotate; alteration in the quaternary structure of haemoglobin is responsible for the sigmoid oxygen dissociation curve, the Bohr effect and the variation of oxygen affinity consequent on interaction with 2,3-DPG (see later). Contacts between like chains, $\alpha_1\alpha_2$ and $\beta_1\beta_2$, are also of physiological significance.

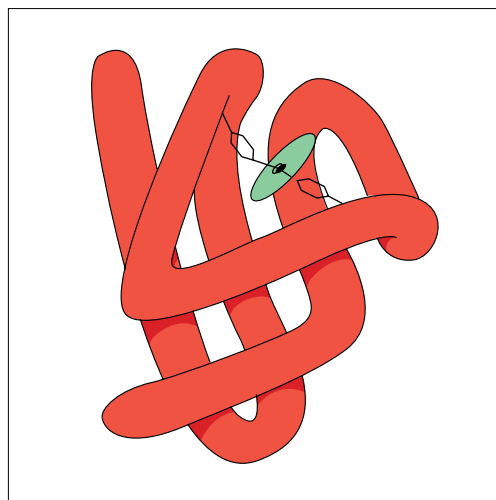


Fig. 1.4 Diagrammatic representation of a haemoglobin molecule with a haem group within the haem pocket, showing the relationship of the haem to two histidine residues of the globin chain, designated proximal and distal histidines; haem is bound to the proximal histidine while O_2 is bound to haem and to the distal histidine, both histidines being important for the integrity of the haem pocket.

The interaction between the four globin chains is such that oxygenation of one haem group alters the shape of the molecule in such a way that oxygenation of other haem groups becomes more likely. This is known as cooperativity and is reflected in the shape of the oxygen dissociation curve (Fig. 1.5). The cooperativity between the globin chains is shown diagrammatically in Fig. 1.6. It is consequent on the fact that in the deoxygenated state the Fe^{2+} atom is out of the plane of the porphyrin ring of haem. Oxygenation of Fe^{2+} causes it to move into the plane of the porphyrin ring and because of the link between haem and the histidine residues of globin there is an alteration in the tertiary structure of that haemoglobin monomer; this in turn causes the oxygenated monomer to alter its position in relation to other haemoglobin monomers, (i.e. the quaternary structure of the haemoglobin molecule is altered). The oxygenated haemoglobin molecule is smaller than the non-oxygenated molecule. Cooperativity between the globin chains is also the basis of the alkaline Bohr effect

(often referred to simply as the Bohr effect) (i.e. the reduction of oxygen affinity that occurs when the pH falls from physiological levels of 7.35 to 7.45 towards 6.0). Increasing metabolism in tissues lowers the pH since there is increased production of CO_2 and of carbonic acid and, in addition, in anaerobic conditions there is generation of lactic acid. The Bohr effect therefore leads to enhanced delivery of oxygen to tissues such as exercising muscle. Similarly, the quaternary structure of haemoglobin makes possible the interaction of haemoglobin with 2,3-DPG, which enhances oxygen delivery. Synthesis of 2,3-DPG is increased by hypoxia. Marked anaemia can cause respiratory alkalosis, which enhances 2,3-DPG synthesis, thus compensating to some extent for the anaemia. There is also increased 2,3-DPG synthesis in renal failure, again partly compensating for the anaemia.

Oxygen affinity is reduced not only by acidosis and increased levels of 2,3-DPG but also by fever. All these effects are likely to be of physiological significance. Fever increases the

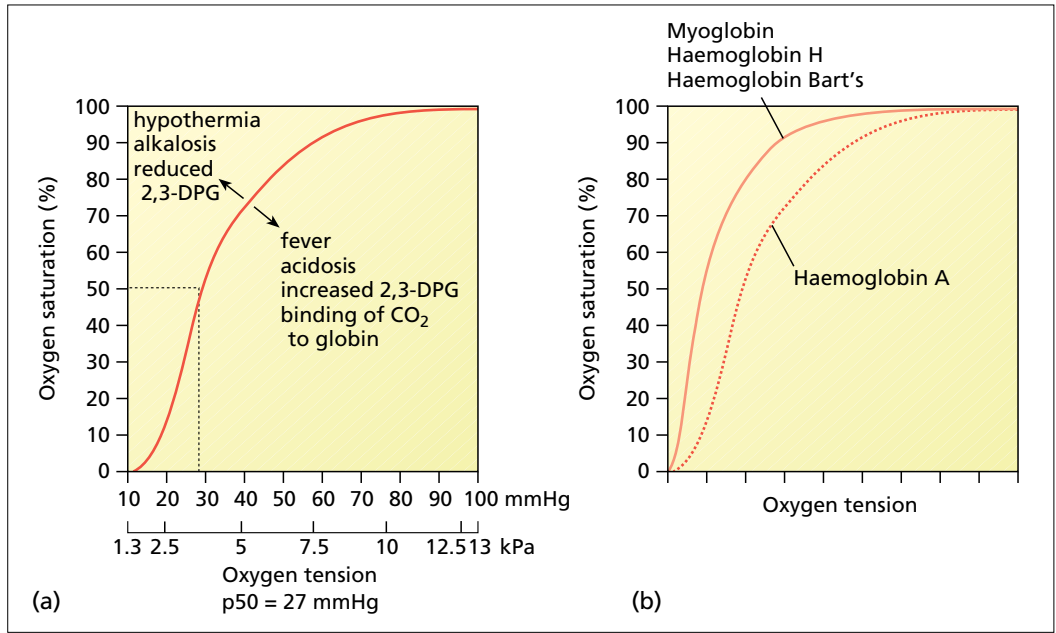


Fig. 1.5 Oxygen dissociation curve: (a) normal oxygen dissociation curve indicating the effects of alteration of pH, body temperature and 2,3-diphosphoglycerate (2,3-DPG) concentration on the oxygen affinity of haemoglobin; (b) a comparison of the hyperbolic oxygen dissociation curve characteristic of myoglobin and of abnormal haemoglobins that do not exhibit cooperativity with the sigmoid dissociation curve characteristic of haemoglobin A; haemoglobin A₂ has a dissociation curve similar to that of haemoglobin A but further to the right.

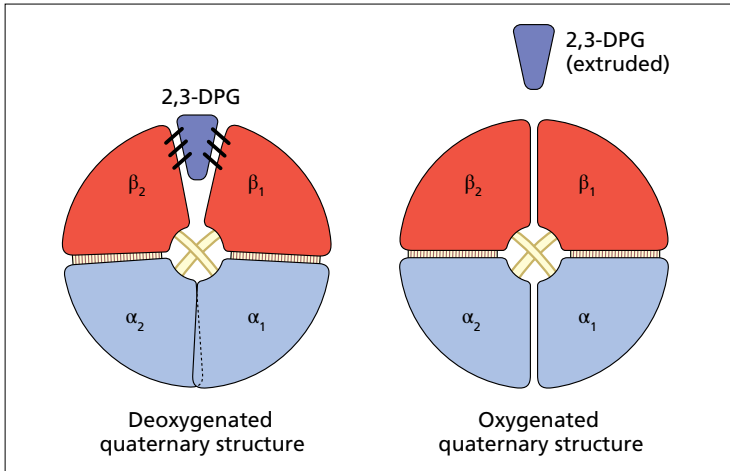


Fig. 1.6 Diagrammatic representation of the effect of oxygenation and deoxygenation on the quaternary structure of haemoglobin. The haemoglobin dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) are stable, with the dimeric bonds between the α and the β chain having 34 contacts in both the deoxygenated and oxygenated forms. There are less strong $\alpha_2\beta_1$ and $\alpha_1\beta_2$ tetrameric bonds, with 17 contacts between the α chain and the β chain, in the deoxy form and a different 17 contacts in the oxy form. There are also $\alpha_1\alpha_2$ bonds with four inter-chain contacts in the deoxy form only. 2,3-DPG binds to the β chains (3 contacts with each chain) only in the deoxy form of the molecule. Oxygenation is associated with breaking and reforming of tetrameric ($\alpha_2\beta_1$ and $\alpha_1\beta_2$) contacts, breaking of $\alpha_1\alpha_2$ contacts, expulsion of 2,3-DPG and the assumption of a more compact form of the molecule. In the deoxygenated form the α chains are closer together and there is a cleft between the β chains whereas in the oxygenated form the α chains are further apart and the β cleft has disappeared.

metabolic rate so that decreased oxygen affinity, favouring offloading of O_2 , is beneficial in this circumstance. The lower pH in tissues favours delivery of oxygen to sites of active metabolism, whereas the efflux of CO_2 in the lungs raises the pH and favours uptake of oxygen by haemoglobin. The oxygen dissociation curve is often right shifted, as a result of acidosis, in chronic renal failure; this ameliorates the effect of anaemia [20]. It will be noted that the acute effect of acidosis and the chronic effect of respiratory alkalosis both contribute to improved oxygen delivery to tissues.

Genetics of haemoglobin synthesis

Haem synthesis takes place in erythroid precursors from the proerythroblast stage to the reticulocyte stage. Eight enzymes, each under separate genetic control, are known to be necessary for haem synthesis [21]. Different stages of haem synthesis take place either in mitochondria or within the cytosol (Fig. 1.7). The

first enzymatic reaction and the last three are in the mitochondrion whereas the four intermediate enzymatic reactions occur in the cytosol. The first rate-limiting step in haem synthesis is formation of δ -aminolaevulinic acid by condensation of glycine and succinyl CoA. This reaction is under the control of aminolaevulinic synthase (ala-synthase) with pyridoxal 5'-phosphate as a cofactor. In erythroid tissue the rate of formation of δ -aminolaevulinic acid is controlled by iron availability; iron deficiency causes iron regulatory proteins to bind to iron-responsive elements in the messenger ribonucleic acid (mRNA) for ala-synthase with resultant repression of translation. Synthesis of δ -amino laevulinic acid is followed by its entry into the cytosol where two molecules combine, under the influence of δ -aminolaevulinic dehydratase (ala-dehydratase), to form porphobilinogen. Four molecules of porphobilinogen in turn combine to form uroporphyrinogen III, which is then modified in two further steps to form coproporphyrinogen III. Coproporphyrinogen

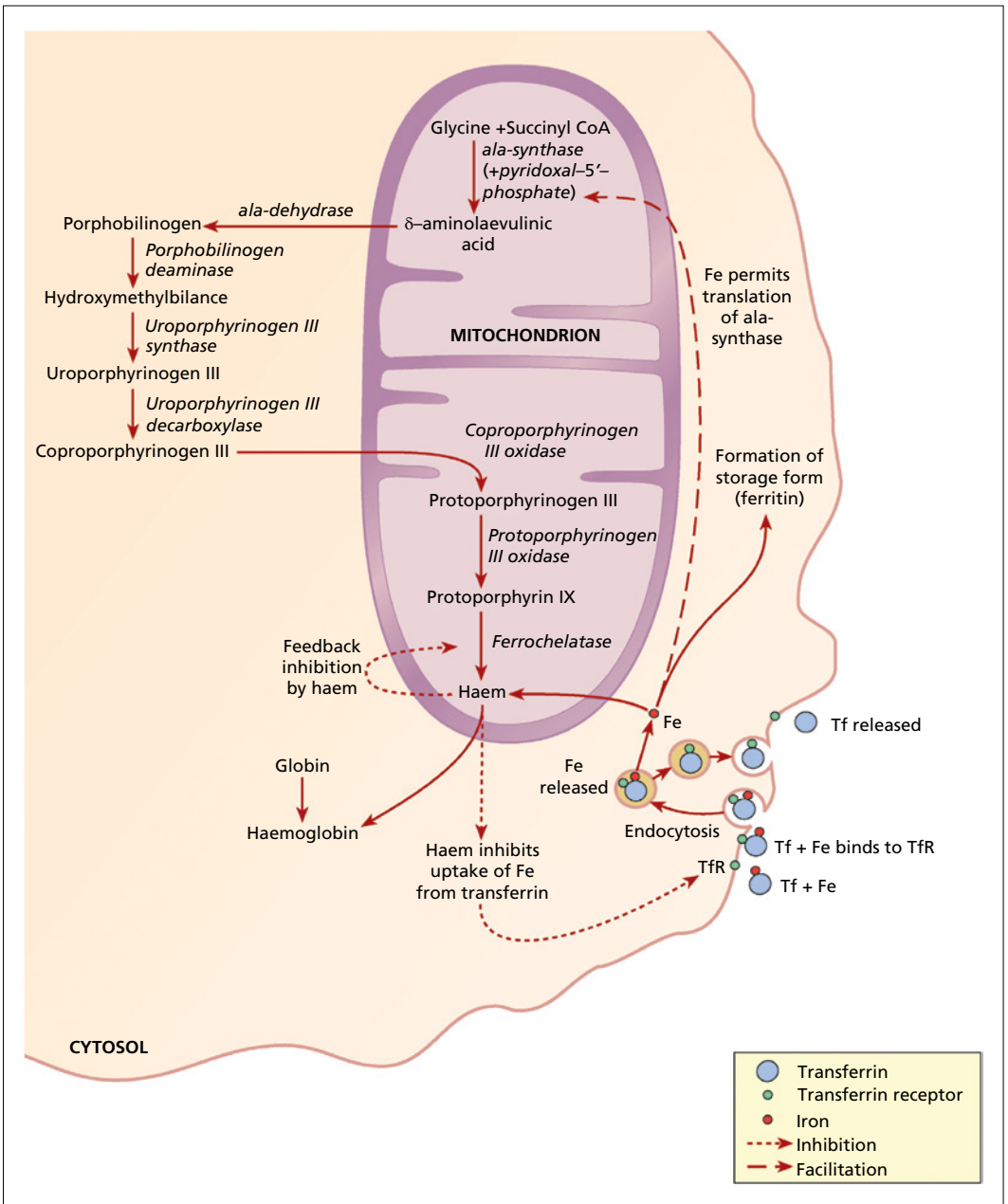


Fig. 1.7 Diagrammatic representation of haem synthesis. Tf, transferrin; TfR, transferrin receptor.

III enters the mitochondrion where it is converted to protoporphyrin IX. The final stage is the combination of ferrous (Fe^{2+}) iron with protoporphyrin IX to form haem, under the influence of ferrochelatase. Haem is also referred to as ferroprotoporphyrin.

Uptake of iron by erythroid cells is from transferrin (see Fig. 1.7). A molecule of transferrin with its attached iron first binds to a membrane transferrin receptor. The whole complex is internalised, in a process known as endocytosis. Iron is released from its carrier

within the endocytotic vesicle and, following reduction to the ferrous form, is either transferred to the mitochondrion for haem synthesis or is stored as ferritin within the cytoplasm. The transferrin molecule then detaches from the transferrin receptor and is released from the cell surface. There is negative feedback control of haem synthesis by haem, which both inhibits ferrochelatase and inhibits acquisition of iron from transferrin. Reduced cellular uptake of iron in turn inhibits production of δ -amino laevulinic acid. Uptake of iron by erythroid cells is enhanced by iron deficiency and by increased levels of erythropoietin. Both lead to combination of iron regulatory proteins with iron-responsive elements in the mRNA for the transferrin receptor protein. The mRNA is then protected from degradation, leading to increased expression of transferrin receptors on erythroid cell membranes and increased iron uptake.

Haem is necessary for normal folding of globin chains and prevents their precipitation [22]. Variant haemoglobins with impaired haem binding are unstable [22]. Haem is important in the regulation of globin chain synthesis. In haem-replete cells a protein known as haem-regulated inhibitor (HRI) is inactive, with the result that guanosine diphosphate (GDP) attached to an erythroid initiation factor, eIF2, is converted to guanosine triphosphate (GTP), leading to initiation of globin chain

synthesis. When haem is deficient, HRI is activated by autophosphorylation and maintains eIF2–GDP in an inactive form so that globin chain translation is not initiated [22]. HRI is likely to be of relevance in β thalassaemia, with increased levels resulting from oxidative damage lessening the excess α chain synthesis.

Synthesis of α , β and γ globin chains takes place in erythroid precursors, from the pro-erythroblast onwards, and continues to the reticulocyte stage. Synthesis of δ chains ceases before the reticulocyte stage [23]. Haemoglobin A synthesis thus continues in reticulocytes, whereas synthesis of haemoglobin A₂ has been completed by the late erythroblast stage [24].

Globin chain synthesis takes place on ribosomes in the cytoplasm. Genes controlling globin chain synthesis are located in two clusters, on chromosomes 11 and 16 (Figs 1.8 and 1.9). The α gene cluster is close to the telomere of chromosome 16, at 16p13.3. The distance from the telomere shows polymorphic variation, from 170 to 430 kilobases (kb). The β gene is at 11p15.4. In addition to the functional globin genes these clusters contain 'pseudogenes', which are non-functional homologues of globin genes; they are transcribed but not translated. The α cluster of chromosome 16 extends over 28 kb and contains, in the following order: a ζ gene, *HBZ*, (also referred to as ζ_2); a pseudo- ζ gene ($\psi\zeta$ or $\psi\zeta_1$); two pseudo- α genes ($\psi\alpha_2$ and $\psi\alpha_1$); and two α

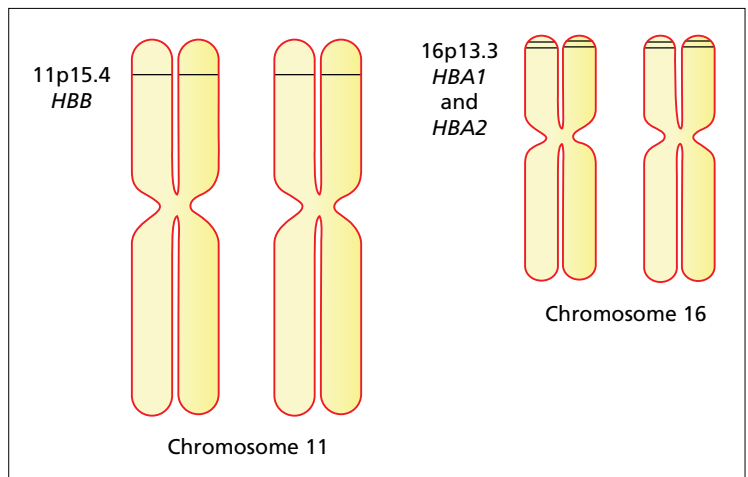


Fig. 1.8 Diagram of chromosomes 11 and 16 showing the positions of the β and α globin gene clusters.

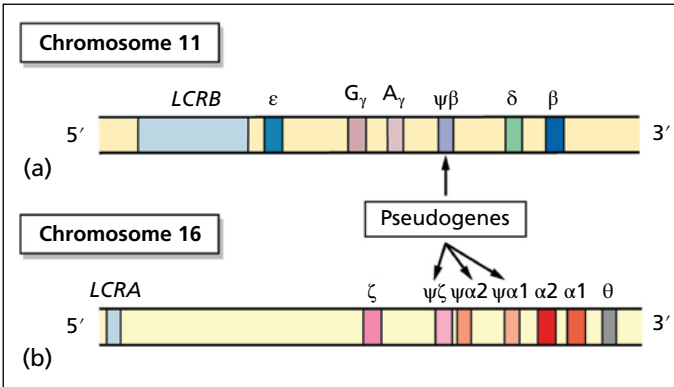


Fig. 1.9 Diagrammatic representation of the α and β globin gene clusters on chromosome 11 (a) and chromosome 16 (b).

genes, *HBA2* and *HBA1*, usually designated $\alpha 2$ and $\alpha 1$. The β cluster on chromosome 11 contains, in the following order: an ϵ gene, *HBE1*; two γ genes, *HBG2* and *HBG1*, usually designated G_γ and A_γ respectively; a pseudo- β gene ($\psi\beta$); a δ gene, *HBD*; and a β gene, *HBB*. There is wide variability of the α and β globin gene clusters between individuals and groups with duplications and triplications of ζ , $\psi\zeta$ and α being quite common. The overall structure of the two clusters are remarkably conserved amongst vertebrates and this has led to the hypothesis that all the globin genes, as well as the gene for the unlinked but related protein, myoglobin, arose from a common ancestor by the processes of duplication, unequal crossing over and sequence divergence. Many primitive invertebrates have only a single globin gene whereas fish and amphibians have an α and a β gene on the same chromosome. Birds have α and β genes on different chromosomes. All the human globin genes have three coding sequences (exons) and two intervening non-coding sequences (intervening sequences or introns) and are flanked by 5' and 3' non-coding sequences (referred to as untranslated regions, UTRs) (Fig. 1.10). The two α genes differ in structure in intron 2 and the 3' UTR but the coding sequences are identical. As for all genes, coding is by means of triplets of nucleotides, known as codons, which code for a specific amino acid. 5' to each gene is the promoter, promoters being sequences that bind ribonucleic acid (RNA) polymerase and transcription factors and are necessary for the initiation of

transcription. Globin gene promoters share several conserved deoxyribonucleic acid (DNA) sequences that bind crucial transcription factors [25, 26]. These are summarised in Table 1.2.

The process by which globin chains are synthesised is shown diagrammatically in Fig. 1.10. Transcription is the process by which RNA is synthesised from a DNA template by the action of RNA polymerase. The entire globin gene, including the introns and the 5' and 3' UTRs, is transcribed. Transcription is controlled by interaction between the genes and transcription factors that bind both to promoters and to upstream regulatory elements referred to as the β -locus control region (*LCRB*) for the β cluster and the α -locus control region (*LCRA*) for the α cluster. The *LCRA* has four regulatory elements, DNase sites HS -48, HS -40, HS -33 and HS -10, also designated R1, R2, R3 and R4, of which HS -40 (R2) is the major regulatory element. It has been estimated that these enhancer elements contribute ~10%, 90%, <2–3% and <2–3% respectively [27]. The *LCRB* includes five erythroid-specific DNase sites designated HS1, HS2, HS3, HS4 and HS5 of which HS3 is probably the most important in opening the chromatin structure to permit access of transcription factors and HS2 is probably the most important in enhancing globin chain synthesis [28]. There are also enhancers and facilitators [29] within introns of genes and downstream of the β and A_γ genes. *Trans*-acting factors, encoded by genes on chromosomes other than 11 and 16, are vital for

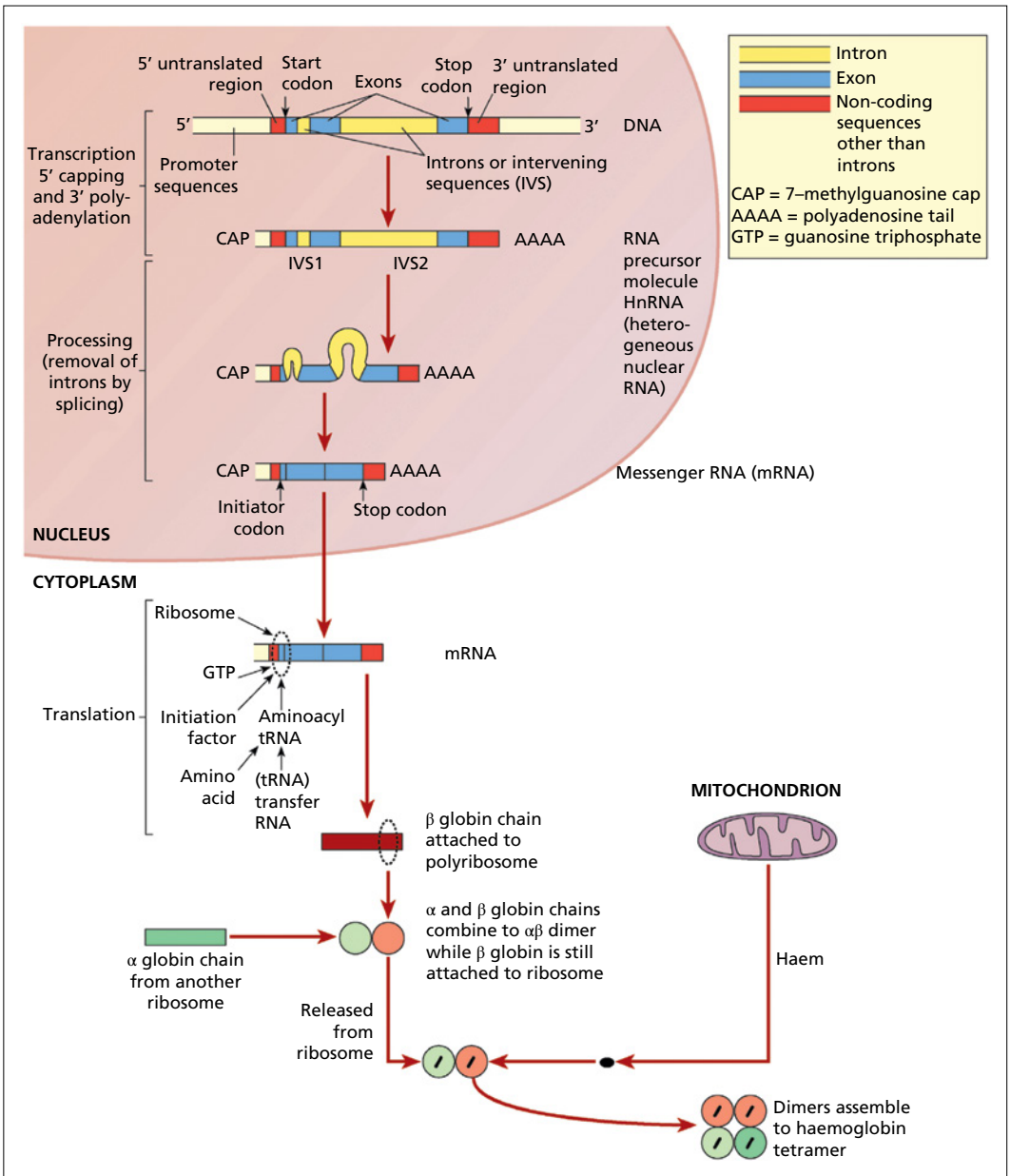


Fig. 1.10 Diagrammatic representation of ribonucleic acid (RNA) synthesis and processing and β globin chain synthesis. Although processes are shown sequentially, capping starts soon after transcription has started and therefore contemporaneously with transcription whereas polyadenylation necessarily occurs after completion of transcription.

the expression of globin genes. Relatively erythroid-specific *trans*-activating factors, including GATA1, ZFPM1 (previously known as FOG1) (which interacts with GATA1 in

erythroid and megakaryocytic development), NFE2, KLF1 (previously known as EKLF), KLF2, NFE4 (SSP), Nrf-1, NFE2L2 (Nrf-2) and NFE2L1 (LCR-F1), contribute to regulation of

Table 1.2 The sequences showing CACCC, CCAAT and TATA homology in the promoters of globin genes; identical sequences in different genes are shown in bold red.

Gene	CACCC homology box	CCAAT homology box	TATA homology box
ζ (<i>HBZ</i>)		CCAAT	T ATA AAC
$\alpha 1$ and $\alpha 2$ (<i>HBA1</i> and <i>HBA2</i>)		CCAAT	C ATA AAC
ϵ (<i>HBE1</i>)		CCAAT	A ATA AAG
γ and δ (<i>HBG2</i> and <i>HBG1</i>)	CACCC	CCAAT/CCAAT	A ATA AAA
β (<i>HBB</i>)	CACCC	CCAAT	C ATA AAA
δ (<i>HBD</i>)		CCAAC	C ATA AAA

gene expression by interacting either with the locus control regions or with the globin gene promoters to increase gene expression [30, 31]. These transcription factors interact, together with many other unidentified factors, in a complex and only partly understood way. KLF1 (Krüppel-like factor 1) is an enhancer of β chain synthesis and a repressor of γ chain synthesis, by means of its activation of BCL11A. BCL11A is part of a repressor complex, also including GATA1 and histone deacetylase 1 (HDAC1), which binds to a region near the 5' end of the δ -globin gene, repressing the gene; this interaction is critical in the fetal-to-adult haemoglobin switch and in γ -globin gene silencing in adults [32]. Heterozygous inactivating mutations of *KLF1* can lead to an increased percentage of haemoglobin F [33] as can haploinsufficiency or downregulation of *BCL11A* [34]. Inactivating mutation of *KLF1* can also lead to an increase of haemoglobin A₂ [35] together with other red cell abnormalities including microcytosis and pyruvate kinase deficiency. SSP (stage selector protein) is an enhancer of δ and γ chain synthesis [30]. NFE4p22 is an enhancer of γ and δ genes [36]. FKL1 and FKL2 are enhancers of the embryonic ϵ gene and the γ genes [36, 37]. *SUPT5H* encodes a putative transactivating factor and mutation can lead to β thalassaemia [38]. In addition to transcription factors that are relatively specific to erythroid cells, globin gene expression is also influenced by general transcription factors including AP-1 (subunits encoded by various genes), Sp1, YY1, USF1 (USF) and TAL-1

(SCL) [28, 30, 31]. Expression of the genes of the β cluster is also influenced by histone acetylases, which increase expression [36]. The γ and δ genes are repressed by histone deacetylases and histone deacetylase inhibitors such as butyrate upregulate γ gene expression [36]. Methylation of genes reduces expression and thus the demethylating action of azacitidine may be the mechanism by which it upregulates γ gene expression [36].

Nascent RNA molecules resulting from transcription are large, unstable and modified in the nucleus. Initially the 5' end acquires a 7-methyl guanosine cap (CAP), which is probably added early during transcription, protects the 5' end of the molecule from degradation and is required for initiation of translation; during this 'capping' process methylation of adjacent ribose residues also occurs. Following the completion of transcription, the majority of transcripts acquire a 3' polyadenosine tail with the addition of 75 to several hundred adenylate residues. There is an AAUAA sequence near the 3' end (within the 3' UTR), which serves as a signal for 3' cleaving of the transcript and polyadenylation. The polyadenylate tail is important for mRNA stability, provides a signal for transfer of mRNA from the nucleus to the cytoplasm and probably enhances translation. Finally the introns are excised to give a functional mRNA, which in most cases contains a single continuous open reading frame (ORF), encoding the sequence of the relevant protein, flanked by 5' and 3' UTRs.

Molecules of mRNA move from the nucleus to the cytoplasm where they bind to ribosomes and

serve as templates for the assembly of the polypeptide sequences of the globin chain. Each nucleotide triplet serves as a template for a specific amino acid that is covalently bound to and transported to the ribosome by transfer RNA (tRNA). tRNAs are specific both for a nucleotide triplet and for an amino acid. Amino acids are thus assembled in the correct sequence, and are covalently bound to each other by ribosomal enzymes, forming a polypeptide. This process is known as translation. An initiation codon, AUG, is essential for the initiation of translation; it is the first codon after the 5' untranslated region and encodes methionine. Initiation requires the amino acid methionine, tRNA specific for methionine, GTP and an initiation factor. When the nascent molecule reaches 20–30 amino acid residues, the methionine is removed through the action of methionine aminopeptidase; this process is interfered with when mutation leads to the presence of certain residues in position 1 or even position 2 of the globin chain [39]. When the chain reaches 40–50 residues co-translational acetylation of the N-terminal residue can occur through the action of several acetyl transferases [40]. Whether this occurs to any great extent depends on the nature of the N-terminal residue. Thus the glycine of the γ chain is 10–15% acetylated whereas the valine of normal α , β and δ chains is resistant to acetylation. Rarely an aberrant amino acid residue present as a result of mutation leads to increased acetylation, as is the case in haemoglobin Raleigh [39]. There are 64 possible nucleotide triplets or codons, 61 of which encode amino acids (20 in all) and three of which do not; the latter serve as STOP or termination codons, leading to termination of globin chain synthesis. Transcription thus continues until a termination codon, UAA, UAG or UGA, is encountered. The termination codon is followed by the 3' UTR.

The rate-limiting step of globin chain translation is the commencement of elongation, which is the next step after initiation. Transcription from the two α genes is equal up to the 8th week of gestation but thereafter the $\alpha 2$ gene becomes dominant and, in adult life, the ratio of $\alpha 2$ to $\alpha 1$ mRNA is 2.6–2.8:1 [41]. Translational

efficiency differs somewhat so that the $\alpha 2$ gene directs synthesis of about twice as much α chain as the $\alpha 1$ gene. There is more α than β mRNA, probably about two and a half times as much, but β chain synthesis is more translationally efficient than α chain synthesis and α chains are therefore produced only slightly in excess of β chains [41]. Control of globin chain synthesis is probably mainly at the level of transcription with translational control being less important. Translation is dependent on the presence of haem. In iron deficiency, reduced availability of haem leads to inactivation of the initiation factor and thus reduced synthesis of globin chains. The α and β globin chains are synthesised on different polyribosomes. Combination of free α chain with β chain that is still attached to the polyribosome, to form an $\alpha\beta$ dimer, may contribute to release of the β chain from the ribosome. Incorporation of haem probably occurs after release from the polyribosome.

Globin mRNA is unusually stable so that translation can continue for up to 3 days after cessation of transcription, and approximately 20% of the total amount of erythrocyte globin is synthesised in the anucleate reticulocyte after its release from the bone marrow. Both the α and β globin genes have structural determinants in their 3' UTRs that are important for mRNA stability [30].

Normal haemoglobins

The normal haemoglobins beyond the neonatal period are haemoglobin A and two minor haemoglobins, haemoglobin A₂ and haemoglobin F.

Haemoglobin A₂

In haematologically normal adults, haemoglobin A₂ comprises about 2–3.5% of total haemoglobin. The percentage is much lower at birth, about 0.2 to 0.3% or even lower, with a rise to adult levels during the first 2 years of life. The steepest rise occurs in the first year but there is a continuing slow rise up to 3 years of age [42] (Fig. 1.11). In the normal adult population, the percentage of haemoglobin A₂ shows a Gaussian distribution. It has functional properties that are

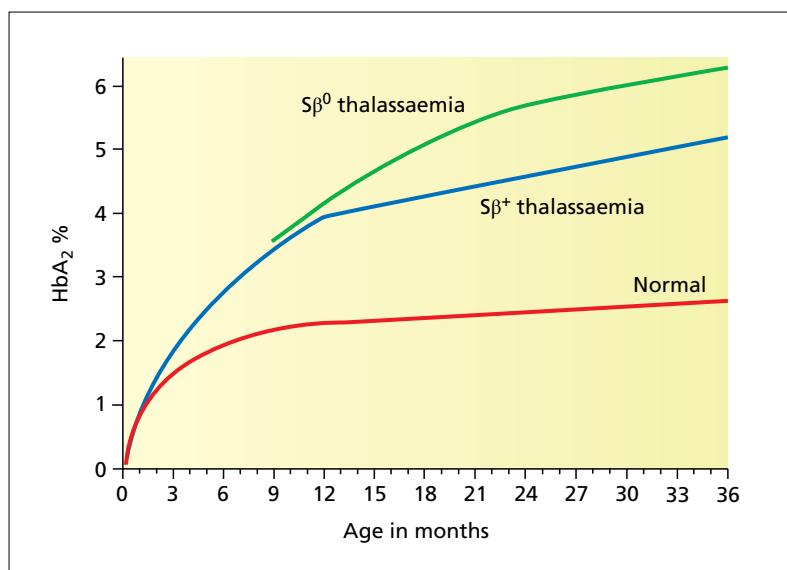


Fig. 1.11 Diagram showing the rate of rise of haemoglobin A₂ in haematologically normal Jamaican babies and in babies with sickle cell/ β thalassaemia. (Adapted from reference [37].)

very similar to those of haemoglobin A [23] (similar cooperativity and interaction with 2,3-DPG) although, in comparison with haemoglobin A, it inhibits polymerisation of haemoglobin S [43] and has a higher oxygen affinity [14]. It has a pancellular distribution.

The reduced rate of synthesis of haemoglobin A₂, in comparison with haemoglobin A, reflects the much slower rate of synthesis of the δ chain in comparison with the β chain. This in turn appears to be consequent in part on a reduced rate of transcription of δ mRNA caused by a difference in the promoter region of these two genes; the δ gene has a CCAAC box rather than the CCAAT box of the β gene [23] and in addition lacks the CACCC sequence that is present in the β promoter (see Table 1.2). In addition, γ chain messenger RNA is unstable and there is also an influence of sequences in IVS2 [44]. The haemoglobin A₂ percentage in adults is controlled by two different genetic mechanisms [45]. Analysis of single nucleotide polymorphisms (SNPs) shows that alleles in the region of the *HBS1L* and *MYB* loci at 6q23.3 influence both the percentage of F cells and the A₂ percentage while alleles around the *HBB* locus at 11p15.4 influence the percentage of haemoglobin A₂ [45]; however, it does not appear to be the *HBS1L* gene itself that is responsible [46]. Inactivating mutations in *KLF1* can cause a borderline to moderate increase in haemoglobin

A₂, up to 3.5–4.7%, with red cell indices typical of β thalassaemia heterozygosity [34, 35, 47, 48]. The proportion of haemoglobin A₂ is slightly reduced by absolute or functional iron deficiency (see Table 6.3) and by α , δ and $\delta\beta$ thalassaemia. In $\gamma\delta\beta$ thalassaemia, the rate of synthesis, but not the proportion of haemoglobin A₂, is reduced since synthesis of γ and β chains is reduced, as well as δ chain synthesis. The proportion of haemoglobin A₂ is increased in the great majority of patients with β thalassaemia trait, in the majority of patient with haemoglobin E heterozygosity [49] and in some patients with an unstable haemoglobin.

Many δ chain variants and δ thalassaemias occur, at least 90 and 25 respectively having been described; some of the δ chain variants are thalassaemic variants. Around 1–2% of individuals of African ancestry have the variant haemoglobin designated haemoglobin A₂' (A₂ prime) or haemoglobin B₂ ($\delta^{16\text{Gly} \rightarrow \text{Arg}}$). In one study, it was the most common haemoglobin variant detected after haemoglobins S and C [44]. Haemoglobin A₂' is readily detected on high performance liquid chromatography (HPLC) (Fig. 1.12) and isoelectric focusing, and may also be detected on capillary electrophoresis (see Fig. 2.27). The δ thalassaemias are also common in some ethnic groups, (present in 1% of Sardinians) [14]. Although some δ chain variants are unstable or have increased oxygen affinity, the low percentage

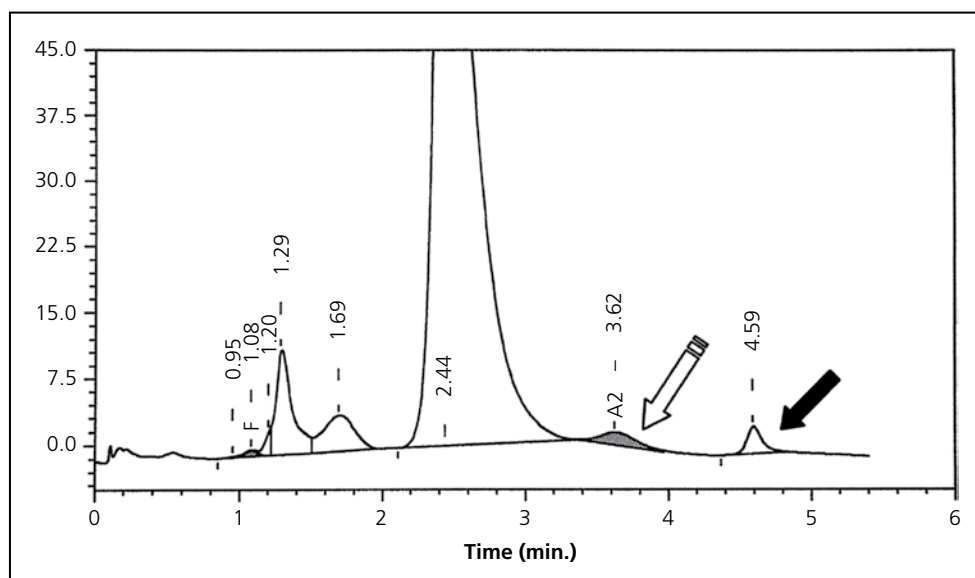


Fig. 1.12 High performance liquid chromatography (HPLC) chromatogram (Bio-Rad Variant II) showing a split haemoglobin A_2 resulting from heterozygosity for haemoglobin A_2' . The white arrow shows haemoglobin A_2 and the black arrow haemoglobin A_2' .

of haemoglobin A_2 means that δ thalassaemia and δ chain variants are of no clinical significance. However, their presence complicates the diagnosis of β thalassaemia trait (see page 141).

Haemoglobin F

Haemoglobin F is the major haemoglobin during fetal life. Its oxygen affinity is higher than that of haemoglobin A and this facilitates oxygen transfer from the mother to the fetus. However, it should be noted that fetal development can be normal in the offspring of mothers with very high levels of haemoglobin F [50]. Its oxygen dissociation curve is sigmoid. The increased oxygen affinity, in comparison with haemoglobin A, is attributable to its weak affinity for 2,3-DPG [10]. In comparison with haemoglobin A, haemoglobin F is less efficient at transporting CO_2 . A significant proportion of haemoglobin F is acetylated.

During the first 2 years of life the haemoglobin F percentage falls progressively to values close to adult levels (Fig. 1.13) [51–53]. A slower fall to final adult levels may continue for several years, even up to puberty and beyond. The percentage of fetal haemoglobin present at birth is quite variable, usually being between 60% and 95%.

During intrauterine life and at birth haemoglobin F shows a C_γ to A_γ ratio of approximately 2:1 to 3:1. Within the first few months of birth this changes to the adult ratio of approximately 2:3. In premature infants there is initially a plateau phase in haemoglobin F percentage lasting 20–60 days followed by a linear decrease similar to that in term babies [52]. At any given period after birth the spread of values is greater than in term babies. Initially there are more high values but after the first month of life values both higher and lower than those of term infants are observed [52]. Haemoglobin F levels at birth are higher in the babies of diabetic mothers and low-for-birth weight babies whereas in Down syndrome the switch to haemoglobin A is earlier [44].

In normal adults, haemoglobin F is heterogeneously distributed, being found in a subset of erythrocytes designated F cells. The proportion of F cells is closely correlated to the percentage of haemoglobin F and is highly variable. In one study the numbers of F cells ranged from 0.6% to 22% [54], although this varies depending on how the HbF is detected. The haemoglobin F percentage is determined by age, gender (slightly higher in women) and a number of

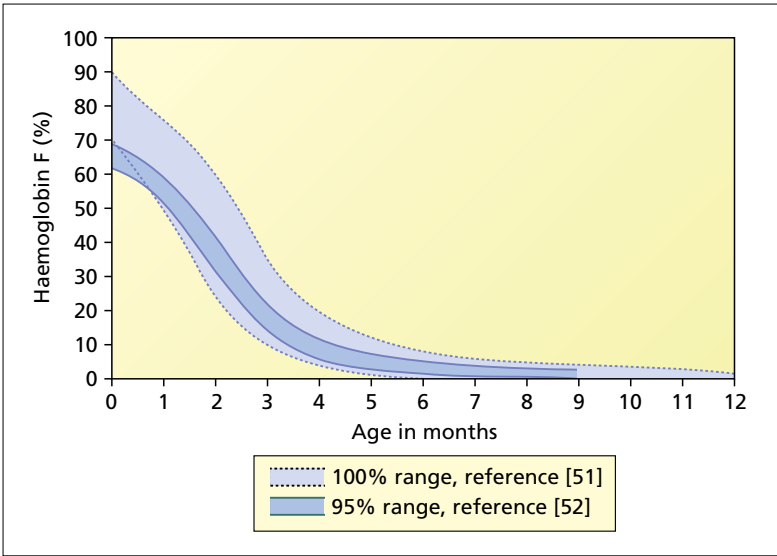


Fig. 1.13 The rate of fall of haemoglobin F percentage postnatally in normal and premature babies; the pale blue areas represent premature babies and the deep blue areas normal babies. (Adapted from references [51, 52].).

inherited characteristics both linked and unlinked to the β globin gene cluster. Variants in three main genes explain 20–50% of the variation in haemoglobin F levels and F cells seen in normal individuals and those with haemoglobinopathies. DNA sequences controlling the proportion of F cells and the percentage of haemoglobin F include [31, 51–58]:

- a polymorphism at position –158 of the $G\gamma$ gene (*HBB2*) (C→T being associated with a higher haemoglobin F) (also known as XmnI $G\gamma$ polymorphism), and largely explains the association between higher haemoglobin F percentage associated with the Senegal and Arab-Indian haplotype in sickle cell anaemia [59], β thalassaemia heterozygosity [60], haemoglobin E heterozygosity [61], haemoglobin E homozygosity [62] and inherited bone marrow failure syndromes [63];
- a *trans*-acting locus at 6q22.3–23.1 in the intergenic region between *HBS1L* and *MYB*, designated haemoglobin F quantitative trait locus 2 (*HBQTL2*) or *HBS1L*-*MYB* intergenic polymorphism block 2 (*HMIP-2*), which affects F-cell numbers and haemoglobin F percentage in haematologically normal Europeans and in individuals with β thalassaemia major and sickle cell anaemia [59] and haemoglobin E homozygosity [62]; *MYB* is a transcription factor for a number of erythroid-specific genes and the intergenic sequences attenuate the function of

nearby enhancers, reducing expression of *MYB* and upregulating haemoglobin F production [64];

- variants in the *BCL11A* gene at 2p16.1, designated haemoglobin F quantitative trait locus 5 (*HBQTL5*), which affect the haemoglobin F percentage in normal Europeans, in individuals with β thalassaemia major and sickle cell anaemia [59], in β thalassaemia heterozygosity [60] and in haemoglobin E heterozygosity [61] and homozygosity [62]; *BCL11A* inhibits γ gene expression, this being of considerable therapeutic importance since gene therapy to downregulate expression can raise the haemoglobin F percentage sufficiently to ameliorate sickle cell anaemia and β thalassaemia.

Many other genetic variants have been identified which are also associated with natural variation in haemoglobin F levels or changes in haemoglobin F expression in experimental systems. These include:

- a *trans*-acting locus at Xp22.2, *FRMPD4* (*FCP1*, *HBQTL3*), which affects F-cell numbers in normal individuals and in males with sickle cell anaemia [59, 65];
- a *trans*-acting locus on chromosome 8q, designated haemoglobin F quantitative trait locus 4 (*HBQTL4*), which may be the *TOX* gene at 8q12.1 [66], which interacts with the common *HBB2* polymorphism (see earlier);
- variation of the number of repeats of a specific motif at –530 in the HS2 component of the β locus control region – (AT)_xN₁₂GT(AT)_y;

- *SAR1A* at 10q22.1, which may influence haemoglobin F response to hydroxycarbamide [59];
- *KLF1* (previously known as *EKLF*) at 19p13.13, (*HBFQTL6*), reduced expression of which leads to reduced expression of the γ gene repressor, *BCL11A*, and therefore increased haemoglobin F; various inactivating heterozygous mutations have led to haemoglobin F levels of 1–3%, 1–7.4% and 3.3–19.5% and a dominant mutation can lead to haemoglobin F level of 40–50% with congenital dyserythropoietic anaemia [33, 67]; compound heterozygosity for S270X nonsense and K332Q mis-sense mutations led to haemoglobin F levels of 22–31%, whereas simple heterozygosity was not associated with an elevation [68];
- expression of *KBX3* (previously *CSDA*) at 12p13.2; cold shock protein domain A is a *trans*-acting suppressor of *HBG2* [69];
- *ZBTB7A* (*LRF*) at 19p13.3 inhibits γ gene expression in adult stage erythroid cells [70];
- *PPARGC1A* at 4p15.2 induces γ gene expression [70];
- the *HBBP1* pseudogene is a negative regulator in adult red cells [71];
- a *BGLT3* long non-encoding RNA is a positive regulator in fetal red cells [72];
- *ANTXR1* at 2p13.3 is associated with reduced haemoglobin F expression with several mutations being associated with a lower haemoglobin F in sickle cell anaemia associated with the Arab-Indian haplotype [73];
- *DNMT1* at 19p13.2 encodes a protein that represses γ gene expression, mutation sometimes leading to amelioration of β thalassaemia [74];
- *BMI1* at 10p12.2 encodes a protein of the polycomb repressor complex that represses haemoglobin F synthesis [75].

There is also a γ -globin gene silencer in a 3.5 kb region near the 5' end of the δ -globin gene which, when deleted, can give rise to hereditary persistence of fetal haemoglobin [32].

The percentage of haemoglobin F is also affected by any increase in the number of γ genes.

The mechanism by which the polymorphisms in the *LCRB* at –530 bp to the *G γ* gene influence γ chain synthesis appears to be that, in comparison with (AT)₇T₇, the (AT)₉T₅ sequence shows increased binding of EIF4EBP1 (BP-1), a negative *trans*-acting factor [76].

The distribution of haemoglobin F percentages in the population is skewed. In 85–90% of individuals, haemoglobin F is less than 0.6–0.7% and F cells are <4.5% [55, 57]. The other 10–15% of the population have values above these levels. The upper limit of normal is rather arbitrarily taken as 1%. It would probably be more accurate to take 0.6% or 0.7% as the upper limit of normal, excluding the 11% of males and 21% of females who have a slight elevation of the percentage of F cells and the haemoglobin F percentage as an X-linked dominant characteristic [55]. However, since the measurement of a low percentage of haemoglobin F is very imprecise, 1% is a practical upper limit.

Haemoglobin F is more markedly increased in patients with various inherited abnormalities of β globin chain synthesis, particularly the more severe forms of β thalassaemia and sickle cell disease, although these high levels are more related to the survival advantages of erythroid cells containing more haemoglobin F, rather than to direct stimulation of γ globin transcription (see Table 3.13). High levels are seen also in various acquired conditions (see Table 6.2).

Haemoglobin F can rise during pregnancy, usually not above 4% [77, 78].

Mutations in γ genes can lead not only to an increased percentage of haemoglobin F but also to haemoglobin F variants, at least 113 of which have been described.

Variant haemoglobins and abnormalities of globin chain synthesis

Nuclear DNA, including the DNA of globin genes, is subject to spontaneous mutation. This may be a point mutation (alteration of a single nucleotide) or a more extensive mutation, in which there is deletion, insertion or other alteration of more than one nucleotide. The types of mutation that can occur in globin genes are summarised in Table 1.3 [79–82]. In addition, expression of globin genes can be affected by DNA sequences outside the globin genes themselves, either enhancers acting in *cis* or genes on other chromosomes encoding *trans*-acting transcription factors (Tables 1.3 and 1.4 [34, 47, 48, 83–86]). The phenotype in

Table 1.3 Types of mutation that can occur in globin genes and adjoining sequences.

Type of mutation	Possible consequence	Examples
POINT MUTATIONS		
Within coding sequence, i.e. within an exon	Same-sense or neutral mutation , i.e. mutant codon codes for same amino acid as normal codon so there are no consequences	Many mutations are of this type; more than a third of theoretically possible point mutations would result in no alteration in the amino acid encoded
	Mis-sense mutation , i.e. mutant codon codes for a different amino acid from the normal codon; includes mis-sense mutations in which an abnormal amino acid interferes with the normal cleavage of the N-terminal methionine	Haemoglobin S, haemoglobin C, haemoglobin E; haemoglobin Marseille and haemoglobin South Florida (altered amino acid near N-terminus plus persisting methionine residue at the N-terminus of the β chain)
	Nonsense mutation , i.e. the mutant codon does not code for an amino acid and thus functions as a STOP or TERMINATION codon, producing a shortened globin chain	Haemoglobin McKees Rocks (two amino acids shorter than normal); $\alpha 2$ CD 116 GAG \rightarrow TAG creating premature STOP codon and causing α thalassaemia
	New-sense mutation , i.e. conversion of a STOP codon to a coding sequence producing an elongated globin chain	Haemoglobin Constant Spring, haemoglobin Icaria, haemoglobin Seal Rock, haemoglobin Koya Dora, haemoglobin Paksé, haemoglobin Zunyi
	Mutation of the initiation codon	α or β thalassaemic disorder
	Gene conversion*	Conversion of $^G\gamma$ gene to $^A\gamma$ gene, giving $^A\gamma^A\gamma$ genotype
		Conversion of $^A\gamma$ gene to $^G\gamma$ gene, giving $^G\gamma^G\gamma$ genotype
		Conversion of $\psi\zeta 1$ to a gene that resembles $\zeta 2$ but is still non-functional
		Gene conversion between the $\alpha 2$ and $\alpha 1$ genes so that the same mutation is present in both, e.g. $\alpha 2^{\text{Lys} \rightarrow \text{Glu}} \alpha 1^{\text{Lys} \rightarrow \text{Glu}}$, giving unusually high levels of haemoglobin I
		Gene conversion between a β gene and a δ gene leading to a haemoglobin A_2 variant (e.g. haemoglobin A_2 Flatbush)
		Haemoglobin F Port Royal, resulting from a further point mutation in a $^G\gamma^G\gamma$ gene complex
		Some β thalassaemias
Within non-coding sequence, i.e. in an intron	Production of a new splice site leading to a structurally abnormal mRNA	

Table 1.3 *Continued.*

Type of mutation	Possible consequence	Examples
Mutation 5' or 3' to the gene (i.e. outside the gene)	Mutation of an enhancer	Some β thalassaemias
	Reduced rate of synthesis of mRNA due to interference with 3'-end formation of mRNA	Some β thalassaemias
DELETION OR DUPLICATION OF ONE OR MORE GENES OR PSEUDOGENES (COPY NUMBER VARIANTS)		
D Deletion of one or more genes	Total loss of expression of relevant gene; occasionally also loss of function of an adjacent structurally normal gene	Most α thalassaemias, some β thalassaemias, $\delta\beta$ thalassaemias and $\gamma\delta\beta$ thalassaemias; deletion of $\epsilon\gamma$ gene ($-\epsilon\gamma$), homozygosity for which causes anaemia and a reduced haemoglobin F percentage in the neonate; deletion of $\psi\zeta 1$
Deletion of genes with downstream enhancer being juxtaposed to remaining gene	Loss of β and δ gene function but enhanced function of remaining $\epsilon\gamma$ ($\pm\epsilon\gamma$) gene	Deletional hereditary persistence of fetal haemoglobin
Duplication of α gene	Triple, quadruple or quintuple α gene	$\alpha\alpha\alpha\uparrow/\alpha\alpha$, $\alpha\alpha\alpha/\alpha\alpha\alpha$, $\alpha\alpha\alpha\uparrow\uparrow/\alpha\alpha$, $\alpha\alpha\alpha\alpha/\alpha\alpha\alpha\alpha$ or $\alpha\alpha/\alpha\alpha\alpha\alpha$
Triplication of entire α globin gene cluster	Six α genes on a single chromosome	$\alpha\alpha:\alpha\alpha:\alpha\alpha/\alpha\alpha$
Duplication of $\epsilon\gamma$ gene	Double, triple or quadruple $\epsilon\gamma$ gene so that there are three, four or five γ genes on a chromosome	$\epsilon\gamma\epsilon\gamma\epsilon\gamma$, $\epsilon\gamma\epsilon\gamma\epsilon\gamma\epsilon\gamma$ (homozygotes have been described with a total of 8 γ genes) or $\epsilon\gamma\epsilon\gamma\epsilon\gamma\epsilon\gamma\epsilon\gamma$
Duplication of the ζ or $\psi\zeta$ gene	Double, triple or quadruple $\zeta/\psi\zeta$ gene	$\zeta 2\psi\zeta 1\psi\zeta 1/\zeta 2\psi\zeta 1$ or $\zeta 2\psi\zeta 1\psi\zeta 1/\zeta 2\psi\zeta 1\psi\zeta 1$ or 4 ζ -like genes per chromosome
ABNORMAL CROSSOVER DURING MEIOSIS LEADING TO GENE FUSION		
$\alpha 2-\alpha 1$ fusion	Effective loss of one α gene but structurally normal α chain is encoded	$-\alpha^{3.7}$ thalassaemia
$\delta\beta$ fusion – simple crossover	Reduced rate of synthesis of structurally abnormal globin chain	Haemoglobin Lepore, e.g. Lepore-Washington/Boston, haemoglobin Lepore-Baltimore and haemoglobin Lepore Hollandia, or $\delta^0\beta^+$ thalassaemia [79]
$\delta\beta\delta$ fusion – double crossover with δ sequences on either side of β sequences	Reduced rate of synthesis of structurally abnormal globin chain	Haemoglobin Parchman
$\beta\delta$ fusion (with preservation of intact δ and β genes on either side of fusion gene, with or without additional mutation)		Anti-Lepore haemoglobins, e.g. haemoglobin Miyada, haemoglobin P-Nilotic, haemoglobin P-Congo haemoglobin Lincoln Park

(Continued on pp. 20–21.)

Table 1.3 *Continued.*

Type of mutation	Possible consequence	Examples
$\Lambda\gamma\beta$ fusion	Synthesis of variant haemoglobin plus increased synthesis of haemoglobin F	Haemoglobin Kenya
$\beta\Lambda\gamma$ fusion (with preservation of intact $\zeta\gamma$ and $\Lambda\gamma$ genes and duplication of the δ gene)		Haemoglobin anti-Kenya
$\zeta\gamma\beta$ fusion	Variant was 37%, A_2 2.4%, F 6.6%, phenotype of β thalassaemia minor	Haemoglobin $\zeta\gamma\beta$ Ulan [80]
$\zeta\gamma\Lambda\gamma$ fusion (designated $-\zeta\gamma\Lambda\gamma-$)	Reduced rate of synthesis of haemoglobin F	γ thalassaemia
DELETION OF DNA SEQUENCES BUT WITHOUT A FRAME SHIFT IN CODING SEQUENCE		
Deletion of part of a coding sequence, either three nucleotides or a multiple of three	One to five amino acids missing but sequence otherwise normal	Haemoglobin Gun Hill (an unstable haemoglobin with five amino acids missing)
DELETION PLUS INVERSION		
Two deletions with inversion of intervening sequence	Deletion involving $A\gamma$ and δ plus β genes respectively but with preservation of an intervening region which is inverted	Indian type of deletional $\Lambda\gamma\delta\beta^0$ thalassaemia
DELETION PLUS INSERTION		
Deletion with insertion of extraneous DNA between breakpoints	Same functional effect as deletion	One type of α^0 thalassaemia, $---^{MED}$
INSERTION WITHIN A CODING SEQUENCE BUT WITHOUT A FRAME SHIFT		
Insertion of nucleotides, either three or multiples of three, e.g. by tandem duplication	Up to five extra amino acids	Haemoglobin Koriyama (an unstable haemoglobin with insertion of five codons in β gene, anti-Gun Hill); haemoglobin Grady (insertion of three codons in α gene)
INSERTION OF A DUPLICATED SEQUENCE INCLUDING THE START CODON		
Tandem duplication of codons flanking the start codon of the <i>HBB</i> gene	Reduced β chain synthesis	β^+ thalassaemia; thalassaemia intermedia in a homozygote [81]
FRAME SHIFT MUTATIONS		
Alteration of the reading frame resulting from deletion, insertion, deletion plus insertion or deletion plus duplication	Abnormal amino acid sequence with an elongated globin chain (when a STOP codon is out of phase and translation continues until another 'in-frame' STOP codon is met); abnormal amino acid sequence with a truncated globin chain (when a premature STOP codon is created)	Haemoglobin Wayne (α chain), haemoglobin Tak (β chain), haemoglobin Cranston (β chain), some β thalassaemias including some dominant β thalassaemias, some α thalassaemias

Table 1.3 *Continued.*

Type of mutation	Possible consequence	Examples
CHROMOSOMAL TRANSLOCATION		
Unbalanced translocation (there may be a balanced translocation in a parent)	Extra α genes on a chromosome other than chromosome 16	Same significance as homozygous triplication of an α gene since there are a total of six α genes
	Loss of an α gene	α thalassaemia; may be part of a contiguous gene syndrome
DELETION OF A LOCUS CONTROL REGION		
Locus control region deleted, with or without deletion of relevant genes	Deletion of the locus control region of the β gene (<i>LCRB</i>)	(ϵ) $\gamma\delta\beta^0$ thalassaemia
	Deletion of the α gene enhancer (HS -40) 40 kb upstream of the $\zeta 2$ gene, also known as MCS-R2 (<i>LCRA</i>)	α^0 thalassaemia
CREATION OF A SEQUENCE THAT COMPETES FOR THE LOCUS CONTROL REGION		
Gain-of-function mutation within α gene cluster	Downregulation by competition with GATA1 for locus control region	α thalassaemia [82]

* Gene conversion is non-reciprocal genetic exchange between allelic or non-allelic homologous sequences so that one gene comes to resemble another more closely or becomes identical to it. This is responsible for maintaining the similarity between pairs of identical or similar genes.

† Either $\alpha\alpha\alpha^{\text{anti3.7}}$ or $\alpha\alpha\alpha^{\text{anti4.2}}$.

‡ Either $\alpha\alpha\alpha^{\text{anti3.7}}$ or $\alpha\alpha\alpha^{\text{anti4.2}}$.

Table 1.4 Mutations and polymorphisms occurring outside the globin gene clusters leading to abnormal globin chain synthesis [34, 47, 48, 83–86].

Mutation	Consequence
Mutation in <i>ATRX</i> gene at Xq21.1 which encodes a <i>trans</i> -acting factor regulating α gene expression	Haemoglobin H disease plus dysmorphism and severe learning difficulties
Mutation in <i>FCP1</i> (<i>HBFQTL3</i>) at Xp22.2	Hereditary persistence of fetal haemoglobin
An <i>HBS1L-MYB</i> intergenic polymorphism at 6q22.3–23.1	Hereditary persistence of fetal haemoglobin
Mutation in the <i>ERCC2</i> (<i>XPD</i>) gene at 19q13.22, which encodes one component of the general transcription factor, TFIIH [83]	Recessive trichothiodystrophy and β thalassaemia
Mutation in the <i>GATA1</i> gene at Xp11.23 [84]	X-linked thrombocytopenia and β thalassaemia (and, in one patient, congenital erythropoietic porphyria) [85]
<i>BCL11A</i> haploinsufficiency (2p16.1)	Increased haemoglobin F associated with cognitive impairment and facial dysmorphism [34]
<i>KLF1</i> at 19p13.13	Increased haemoglobin A ₂ [47, 48]
<i>ASH1L</i> at 1q22	β thalassaemia [86]

such cases can be that of thalassaemia trait or silent thalassaemia [87].

Variants of globin genes sometimes have no effect on the amino acid sequence. This occurs because, as mentioned earlier, there is redundancy in the genetic code, with a number of nucleotide triplets encoding the same amino acid. When a 'same sense' mutation occurs, the new codon resulting from the mutation codes for the same amino acid as the original codon and there is thus no effect on the final gene product. Similarly, mutation of a termination codon may be to a different termination codon. Many spontaneous variants of globin genes are 'same-sense' mutations. Point mutations can also result in a 'mis-sense' mutation when the new codon encodes a different amino acid, leading to production of a variant haemoglobin. The site of a mutation is critical, determining whether there is an effect on stability, oxygen affinity, solubility and other critical characteristics of the haemoglobin molecule. Because of the redundancy in the genetic code, different point mutations can give rise to the same variant haemoglobin. For example, the α chain variant, G-Philadelphia, has arisen twice, from an AAC to AAG change in an $\alpha 2\alpha 1$ fusion gene and from an AAC to AAA change in an $\alpha 2$ gene [88]. There are more than 1000 known variant haemoglobins resulting from point mutations in the α or β genes. There are also at least 40 variant haemoglobins resulting from two point mutations in the same gene with two amino acid substitutions. This can result either from a new mutation occurring in the gene encoding a variant globin chain (e.g. in a parental germ cell) or from crossover between two variant alleles. Usually the second mutation has occurred in a gene that would otherwise encode a relatively common variant haemoglobin such as haemoglobin S, C, E or D-Punjab. Strangely, double mutations have been much more frequently described in the β gene than in the α genes.

Some point mutations are 'nonsense' mutations in which the new codon is one of the three that do not code for an amino acid. A 'nonsense' mutation thus functions as a 'STOP' or 'TERMINATION' codon, leading to termination of chain synthesis. If this type of mutation occurs

towards the 5' end of the gene, no functional protein is produced. However, when the mutation occurs near the 3' end, an abnormal globin gene may be produced which interferes with the formation of functional haemoglobin tetramers and leads to a dominant thalassaemia phenotype. Point mutations can also convert a STOP codon to a coding sequence so that an elongated mRNA and elongated globin chain are produced.

An unusual result of a point mutation is production of an abnormal amino acid that is converted to a different amino acid by post-translational modification. This can be as the result of deamidation, acetylation or oxidation. There are at least six reported variant haemoglobins in which the abnormal DNA sequence codes for asparagine but this is subsequently deamidated to aspartic acid [19]; of these, the most common is haemoglobin J Sardegna ($\alpha 50[\text{CD}8]^{\text{His} \rightarrow \text{Asn} \rightarrow \text{Asp}}$), which has a prevalence of 0.25% in northern Sardinia. Post-translational acetylation occurs in haemoglobin Raleigh, which has a $\beta 1^{\text{Val} \rightarrow \text{Ala}}$ substitution; proteins with an N-terminal alanine are often acetylated and this is the case with this variant haemoglobin [89]. The presence in one individual of haemoglobins with three different β chains may be attributable to post-translational modification. For example, the replacement of leucine by hydroxyleucine that characterises haemoglobin Coventry is not encoded by genomic DNA and is found only in the presence of an unstable haemoglobin, either haemoglobin Atlanta or haemoglobin Sydney. Some mutations affecting the haem pocket and leading to haemoglobin instability permit oxidation of leucine to isoleucine [90]. Haemoglobin Bristol also shows post-translational modification. It is an unstable haemoglobin resulting from conversion of the $\beta 67$ valine codon to a codon for methionine; however, the final haemoglobin has aspartic acid rather than methionine as a result of post-translational modification [19].

In a slightly different mechanism, the abnormal structure of a variant haemoglobin resulting from a point mutation leads to post-translational modification of a normal amino acid, in three cases leucine being modified to hydroxyleucine [19] and in one case asparagine

adjacent to the abnormal residue being deamidated to aspartic acid [89].

Mutations in the codon for the N-terminal valine may mean that a different amino acid is encoded with resultant retention of the initiator methionine and full acetylation of the N-terminal residue (e.g. the glutamate of the α chain variant haemoglobin Thionville) or normal cleavage of methionine but full acetylation of the N-terminal residue (e.g. the alanine of the α chain variant haemoglobin Lyon-Bron) [40]. Similarly, a histidine to proline change in position β 2 leads to retention of the initiator methionine [89]. If methionine is retained, the globin chain is extended by one residue.

Deletions and insertions can lead to a frame shift; unless the deletion or insertion involves three nucleotides or multiples of three the nucleotide sequences beyond the mutation will be in a different reading frame and will be 'read' during translation as coding for a completely different sequence of amino acids. Frame shift mutations can lead to a premature STOP codon

so that both mRNA and the resultant globin chain are shorter than normal. Unless this occurs, a frame shift mutation is likely to lead to elongated mRNA and an elongated globin chain. The original STOP codon is no longer in the reading frame and transcription continues until another STOP codon is encountered.

Small deletions and large deletions and insertions can result from non-homologous crossover between a pair of chromosomes during meiosis. These are usually in-frame. Non-homologous crossover can involve not only a single pair of allelic genes (e.g. two α genes) but also two structurally similar but non-allelic genes (e.g. a β gene and a δ gene); in the latter instance there may be a loss of the two normal genes and production of a fusion gene that has 5' sequences of one gene and 3' sequences of the other gene; alternatively the two normal genes may be retained with part of both genes being reduplicated in the fusion gene. Some examples of non-homologous crossover are shown in Fig. 1.14. Non-homologous crossover can also result in

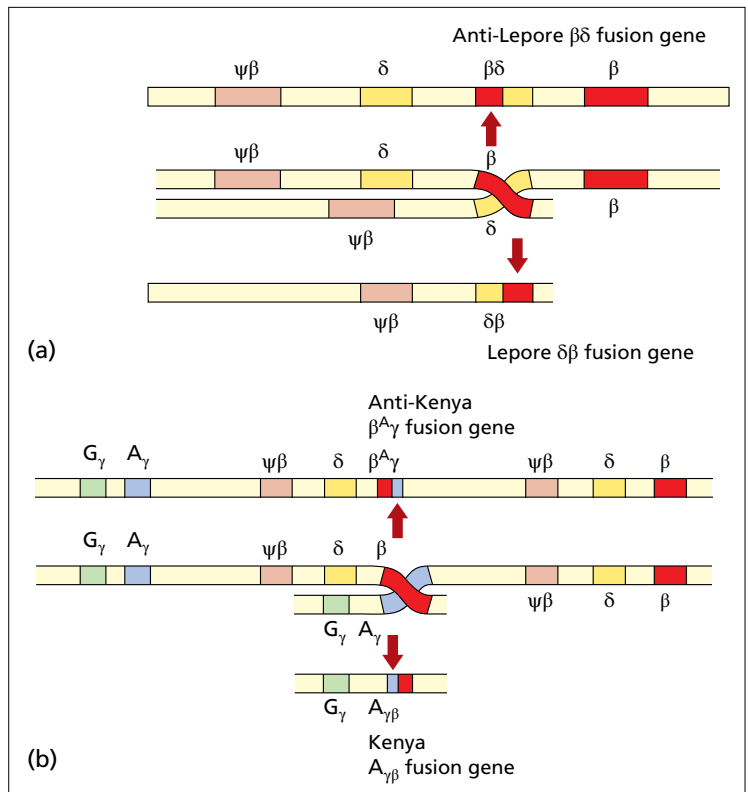


Fig. 1.14 Some examples of fusion genes produced by non-homologous crossover: (a) formation of genes encoding Lepore and anti-Lepore haemoglobins; (b) formation of genes encoding Kenya and anti-Kenya haemoglobins.

the reduplication or loss of some genes, referred to as copy number variants (CNVs); for example, some individuals, instead of having two α genes on each chromosome 16, have three, four or even five [91] α genes on one chromosome. Duplicated α genes occur in many populations and in some are quite frequent. For example, 2% of Sri Lankans have $\alpha\alpha\alpha$.

Very rarely, individuals are somatic mosaics so that a variant haemoglobin is present in an unusually low percentage. For example, haemoglobin Costa Rica, a β chain variant arising as a result of somatic mutation, constituted 5–6% of total haemoglobin in the individual in whom it was recognised [92]. Similarly, a patient has been reported with haemoglobin Korle Bu as a minor fraction as a result of constitutional mosaicism [93].

Haemoglobin dimers are stable but the tetramers that they form are able to dissociate and re-associate. When both normal and variant globin chains are present, heterotetramers and homotetramers will be present *in vivo* (e.g. in the case of sickle cell trait there will be $\alpha_2\beta_2$, $\alpha_2\beta_2^S$ and $\alpha_2\beta\beta^S$). When haemoglobins are studied *in vitro* (e.g. by electrophoresis or chromatography), the heterotetramers dissociate and re-associate as homotetramers. Some variant haemoglobins have abnormally stable tetramers so that three rather than two forms are detected on haemoglobin electrophoresis and similar techniques.

Thalassaemias and variant haemoglobins

Mutations can lead not only to synthesis of a structurally abnormal haemoglobin but also to a reduced rate of synthesis of a globin chain and therefore of the haemoglobin species of which it forms a part. The term 'thalassaemia' is used to describe disorders with a significant decrease in the rate of synthesis of one or more globin chains. α thalassaemia indicates a reduced rate of synthesis of α globin chain. Similarly, β , δ , $\delta\beta$ and $\gamma\delta\beta$ thalassaemias indicate a reduced rate of synthesis of β , δ , $\delta + \beta$ and $\gamma + \delta + \beta$ chains respectively. In some disorders there is both synthesis of a structurally abnormal haemoglobin and a reduced rate of synthesis of the variant

haemoglobin. This is the case, for different reasons, with the α chain variant, haemoglobin Constant Spring (first described in a Chinese patient in Constant Spring, a district of Kingston, Jamaica), and the β chain variant, haemoglobin E. The term 'haemoglobinopathy' is sometimes used to indicate only those disorders with a structurally abnormal haemoglobin, whereas others use the term to include all disorders of globin chain synthesis, encompassing also the thalassaemias. The second use seems preferable since some variant haemoglobins, such as haemoglobin E and haemoglobin Constant Spring, are synthesised at a reduced rate and thus constitute thalassaemic haemoglobinopathies, and some thalassaemias lead to synthesis of a structurally abnormal haemoglobin, such as haemoglobin H or haemoglobin Bart's, as a result of unbalanced chain synthesis.

Haemoglobinopathies can result from mutation of a β globin gene, in which case there is only a variant form of haemoglobin A, or mutation of an α globin gene, in which case there are variant forms of haemoglobins F, A and A_2 . Similarly, mutations of γ or δ genes result in mutant forms of haemoglobin F and haemoglobin A_2 respectively. Because there are two β genes, an individual can have two different β globin variants. Because there are usually four α genes, an individual could, in theory, have up to four different α chain variants; in practice, a number of individuals have been described with many different haemoglobin variants, for example, haemoglobin Buda, haemoglobin Pest and haemoglobin A in one instance and haemoglobin $G^{\text{Philadelphia}}$, haemoglobin J^{Sardigna} and haemoglobin A in several instances. These tend to be more common in countries, such as Thailand, with a high incidence of many different variants.

The proportion of variant haemoglobins

The proportion of an α chain variant in the blood might be expected to be around 25%, since there are usually four α genes. However, the situation is far more complex. The variant is likely to be more than 25% if it results from a mutation of the $\alpha 2$ gene (since the ratio of $\alpha 2$ to $\alpha 1$ synthesis is normally about 3:1) and less

Table 1.5 Consequences of mutations of globin genes.

Type of mutation and consequence	Examples
Substitution of an external amino acid which is not involved in inter-chain contacts; no functional abnormality	Haemoglobin G-Philadelphia
Amino acid substitution leading to reduced solubility, polymerisation of haemoglobin and deformation of cells into a holly leaf or sickle shape with consequent haemolysis and vascular obstruction	Haemoglobin S (sickle cell haemoglobin)
Amino acid substitution leading to reduced solubility, formation of straight-edged crystals and haemolysis	Haemoglobin C
Replacement of haem-binding or haem-related histidine residue by another amino acid leading to an increased tendency to oxidation, i.e. formation of methaemoglobin. There is cyanosis at birth if the defect is in a γ gene, cyanosis from birth if the defect is in an α chain and cyanosis from approximately 6 months of age if the defect is in a β chain. There may be associated haemoglobin instability	M haemoglobins
Mutation involving amino acids of the haem pocket or $\alpha_1\beta_2$ (tetrameric) contacts or mutation interfering with the helical structure of haemoglobin, leading to haemoglobin instability and Heinz body haemolytic anaemia; there may also be decreased oxygen affinity and resultant cyanosis	Haemoglobin Köln, haemoglobin Zurich (haem pocket mutation), haemoglobin Kansas (mutation affecting $\alpha_1\beta_2$ contacts)
Mutations involving $\alpha_1\beta_2$, $\alpha_2\beta_1$ tetrameric haemoglobin contacts or C-terminal end of β chain, where there are residues involved in 2,3-DPG interaction and stability of the deoxy form of haemoglobin, leading to increased oxygen affinity and polycythaemia	Haemoglobin Chesapeake, haemoglobin Bethesda, haemoglobin Kempsey, haemoglobin J-Cape Town, haemoglobin Yakima
Mutation leading to decreased oxygen affinity and therefore anaemia, since normal tissue delivery of oxygen is achieved with a lower concentration of haemoglobin. May cause cyanosis	Haemoglobin S, haemoglobin Seattle (also unstable), haemoglobin Kansas (also unstable), haemoglobin Beth Israel
Mutation in β gene leading to markedly reduced or absent β chain production, reduced synthesis of haemoglobin A and possibly ineffective erythropoiesis consequent on damage to developing erythroblasts by excess α chains	β thalassaemia (major, intermedia or minor)
Mutation in β gene leading to truncated and very unstable β chain	(Dominant) β thalassaemia phenotype
Mutation in one or more α genes leading to markedly reduced or absent α chain synthesis and reduced synthesis of haemoglobins F ₁ and A ₂	α thalassaemia (α thalassaemia trait, haemoglobin H disease or haemoglobin Bart's hydrops fetalis)
Mutation in α gene leading to structurally abnormal α chain synthesised at a greatly reduced rate	α thalassaemia phenotype, e.g. haemoglobin Constant Spring (mRNA and the haemoglobin are unstable)
Mutation in δ gene leading to a structural abnormality or markedly reduced or absent δ chain production	Haemoglobin A ₂ variant or δ thalassaemia. No clinical significance as haemoglobin A ₂ is a minor haemoglobin but complicates the diagnosis of β thalassaemia trait
Mutation in γ gene leading to structural abnormality or reduced rate of synthesis of γ chain and therefore haemoglobin F	Low haemoglobin F levels or haemoglobin F variant

2,3-DPG, 2,3-diphosphoglycerate; mRNA, messenger ribonucleic acid.

than 25% if it results from mutation of an $\alpha 1$ gene. The percentage is raised if there is coinheritorship of α thalassaemia and is lowered if there is coinheritorship of triple α ($\alpha\alpha\alpha$). If a gene encoding an α chain variant is a mutated $\alpha 1$ gene in *cis* with deletion of the $\alpha 2$ gene then it can be upregulated, increasing the percentage further. The percentage is reduced if the variant α chain is synthesised at a reduced rate, if it has a lower affinity for β chains than does the normal α chain or if the variant α chain or the variant haemoglobin is unstable.

Similarly, it might be expected that a β chain variant would be about 50% of total haemoglobin in heterozygotes since there are two β genes. As for α chain variants, the situation is much more complex. The percentage may be above 50% in the case of variants with negatively charged β chains, which have a greater affinity than the normal β chain for the positively charged normal α chains (e.g. haemoglobin J Baltimore or J-Iran); if there is coexisting α thalassaemia, leading to a lack of α chains, the percentage of the variant is even higher. The converse is seen with positively charged β chains, such as β^S , β^C , β^{O-Arab} and $\beta^{D-Punjab}$, which have a lower affinity than normal β chains for normal α chains. The percentage of the variant is thus somewhat less than 50% and if there is coexisting α thalassaemia the percentage is even lower. The percentage is also reduced considerably below 50% if there is a reduced rate of synthesis of the variant β (or $\delta\beta$) chain (e.g. β^E , $\delta\beta^{Leptore}$), if the β chain is unstable or if the variant haemoglobin is unstable (e.g. haemoglobin Köln).

An alteration in the amino acid sequence of the globin chains (an alteration in the primary structure of haemoglobin) often has no significant effect on the secondary, tertiary and quaternary structure of haemoglobin; this is the case when the substituted amino acid is of similar size to the normal amino acid, has the same charge and the same hydrophobic or hydrophilic properties and does not have a role in the binding of haem or 2,3-DPG nor in interactions between chains. This is the case for the majority of variant haemoglobins, which have no consequences for the health of the individual. In other cases an alteration in the primary structure of haemoglobin affects the secondary, tertiary or

quaternary structure of the molecule, sometimes with very profound effects. Some of the effects of mutations in globin genes are shown in Table 1.5.

At least 1870 variants of the globin genes have been identified. Some 690 of them were initially collated in a single volume [94] and this database is now available electronically, in an updated and greatly expanded form (<http://globin.cse.psu.edu/>).

Check your knowledge

One to five answers may be correct. Answers to almost all questions can be found in this chapter or can be deduced from information given. Answers are given on page 32.

- 1.1 The haemoglobin molecule
 - (a) requires iron for its synthesis
 - (b) is composed of three pairs of globin chains
 - (c) alters its structure when oxygen is bound
 - (d) is assembled in the cytosol
 - (e) binds 2,3 diphosphoglycerate
- 1.2 Haemoglobin F
 - (a) is the major haemoglobin present in the fetus
 - (b) has a lower oxygen affinity than haemoglobin A
 - (c) is absent in normal adults
 - (d) percentage shows a non-Gaussian distribution in the population
 - (e) is composed of two α chains and two β chains
- 1.3 The functions of haemoglobin include
 - (a) transport of glucose
 - (b) transport of CO_2
 - (c) transport of O_2
 - (d) buffering
 - (e) transport of creatinine to the kidney
- 1.4 The affinity of haemoglobin for oxygen is decreased by
 - (a) fever
 - (b) metabolic alkalosis
 - (c) binding of CO_2

- (d) binding of 2,3 diphosphoglycerate
 - (e) glycosylation
- 1.5 When blood circulates through the lungs haemoglobin
- (a) is oxidised
 - (b) takes up oxygen
 - (c) loses CO₂
 - (d) takes up water
 - (e) dissociates into haem and globin
- 1.6 Structurally abnormal haemoglobins may result from
- (a) point mutations
 - (b) gene fusion
 - (c) frame shift mutations
 - (d) mutation of STOP codon to a coding sequence
 - (e) mutation of a coding sequence to a STOP codon
- 1.7 Abnormal haemoglobins may
- (a) have increased oxygen affinity
 - (b) have decreased oxygen affinity
 - (c) be prone to crystallise
 - (d) be unstable
 - (e) be abnormally prone to oxidation
- 1.8 Mutations in globin genes
- (a) can occur in α , β , γ , δ genes
 - (b) always result in a structural abnormality of haemoglobin
 - (c) always have harmful effects
 - (d) can lead to a reduced rate of globin chain synthesis
 - (e) can convert one gene to another
- 1.9 Haemoglobin F
- (a) is present, in adult life, in a subset of erythrocytes referred to as F cells
 - (b) is composed of two α chains and two γ chains, encoded by two pairs of structurally similar α genes and two pairs of structurally similar γ genes
 - (c) has a sigmoid dissociation curve
 - (d) constitutes a higher proportion of total haemoglobin in premature than in full-term babies
 - (e) on average is present at a higher level in women than in men
- 1.10 Cooperativity is essential for
- (a) a sigmoid oxygen dissociation curve
 - (b) the higher oxygen affinity of haemoglobin F in comparison with haemoglobin A
 - (c) the Bohr effect
 - (d) the binding of CO₂ to haemoglobin
 - (e) conversion of haemoglobin to methaemoglobin
- 1.11 The proportion of a variant haemoglobin is usually
- (a) greater in the case of an α chain variant than a β chain variant
 - (b) greater in the case of an α chain variant if there is coexisting deletion of an α gene
 - (c) greater if the variant β chain has a higher affinity for normal α chain than does the normal β chain
 - (d) greater, in the case of haemoglobin S, if there is coexisting α thalassaemia
 - (e) greater if the variant haemoglobin is unstable

Further reading and resources

- Bain BJ, Wild BJ, Stephens AD and Phelan LA. *Variant Haemoglobins: A Guide to Identification*. Oxford, Wiley-Blackwell, 2010.
- The Globin Gene Server, hosted by Pennsylvania State University, USA and McMaster University, Canada. <http://globin.cse.psu.edu/>
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Answers to questions

- | | | | | | |
|-----------|-----------|-----------|-----------|------------|------------|
| 1.1 (a) T | 1.3 (a) F | 1.5 (a) F | 1.7 (a) T | 1.9 (a) T | 1.11 (a) F |
| (b) F | (b) T | (b) T | (b) T | (b) T | (b) T |
| (c) T | (c) T | (c) T | (c) T | (c) T | (c) T |
| (d) T | (d) T | (d) F | (d) T | (d) T | (d) F |
| (e) T | (e) F | (e) F | (e) T | (e) T | (e) F |
| 1.2 (a) T | 1.4 (a) T | 1.6 (a) T | 1.8 (a) T | 1.10 (a) T | |
| (b) F | (b) F | (b) T | (b) F | (b) F | |
| (c) F | (c) T | (c) T | (c) F | (c) T | |
| (d) T | (d) T | (d) T | (d) T | (d) F | |
| (e) F | (e) F | (e) T | (e) T | (e) F | |

2 Laboratory techniques for the identification of abnormalities of globin chain synthesis

The diagnosis of disorders of globin chain synthesis usually requires a combination of techniques. It is important to recognise that many of the laboratory tests in routine diagnostic use indicate only the physicochemical characteristics of a haemoglobin rather than permitting its precise identification. For clinical purposes an adequate presumptive identification usually requires a combination of at least two techniques with results being assessed in relation to the clinical features, the ethnic origin of the subject and the blood count and film [1]. Principles of techniques and selection of technique will be discussed in this chapter. Some methods found satisfactory in the laboratories with which the authors are associated are given as an appendix to this chapter (see page 85). For precise technical details and other recommended methods the reader is referred to references [2–4].

Sample collection

Laboratory investigations for haemoglobinopathies are most conveniently performed on venous blood samples anticoagulated with one of the salts of ethylene diamine tetra-acetic acid (e.g. K_2EDTA). In the case of children, anticoagulated capillary samples obtained by skin prick (e.g. from the heel) are also suitable. Testing of neonates can be performed on cord blood, venous blood or skin prick samples. To reduce the chances of maternal contamination, cord blood samples should be obtained from an

umbilical cord vessel by means of a syringe and needle after wiping away any surface blood. They should not be obtained by squeezing blood from the end of the cord. Skin prick samples from neonates can be taken into a heparinised capillary tube and sent to the laboratory as anticoagulated blood or can be absorbed directly onto a filter paper and sent as a dried blood spot, usually referred to as a 'Guthrie spot' from the originator of the technique. Anticoagulated blood is more stable than dried blood spots and the bands obtained on electrophoresis are more distinct.

Samples should be stored at 4°C and ideally should be tested within a week as longer storage leads to denaturation of haemoglobin and less distinct bands on electrophoresis. Dried blood spots are stable for 7–10 days at room temperature.

Samples for testing should be accompanied by information including the full name, gender, date of birth and ethnic origin of the individual to be tested. Knowledge of clinical features and family history is sometimes essential for adequate interpretation and information on parental consanguinity is also useful. When blood is taken for genetic counselling of potential parents, identifying details of the partner should also be given so that results of both partners can be assessed simultaneously and guidance on genetic risks can be given. Those responsible for requesting tests and obtaining blood samples should ensure that samples are not inadvertently obtained after a blood transfusion has been given.

The blood count, film and reticulocyte count

A full blood count (FBC) and a blood film examination are usually indicated whenever an abnormality of globin chain synthesis is suspected. The exception is in neonatal screening for haemoglobinopathies when usually only a small volume of blood, possibly only a dried blood spot, is available for analysis. For workload reasons, a blood film is not usually available in antenatal screening.

The FBC is essential in the assessment of possible thalassaemia and in the differential diagnosis of thalassaemia and hereditary persistence of fetal haemoglobin.

A reticulocyte count is indicated if a blood film shows polychromasia or if haemoglobin H disease or an unstable haemoglobin is suspected (Fig. 2.1).

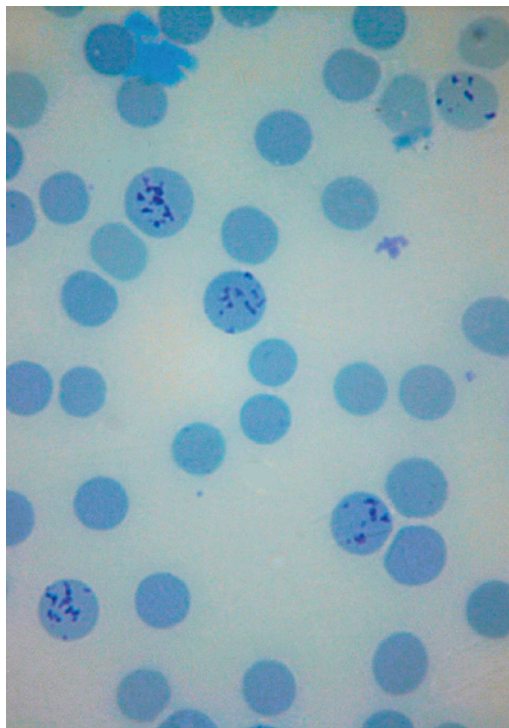


Fig. 2.1 Reticulocyte preparation showing an increased reticulocyte count in a patient with homozygosity for haemoglobin Bushwick, a mildly unstable haemoglobin.

Preparing a red cell lysate

A red cell lysate suitable for most purposes is most simply prepared by adding one drop of red cells to 20 drops of a proprietary reagent (Haemolysate reagent, Helena Biosciences Europe) and standing for two minutes; the lysate is then used for testing. Alternatively, two volumes of packed red cells can be added to one volume of cool distilled water, followed by vigorous mixing on a vortex mixer, standing for two minutes, followed by mixing again, centrifugation (preferably at 4°C) and pipetting off the supernatant for use. Lysates are prone to oxidation so should be used promptly, always within one week. If there is a requirement for long-term storage of a lysate then a solution of toluene is preferred. Carbon tetrachloride has also been used but is banned in some countries. An alternative is to freeze drops of washed red cells by dropping them on to a layer of liquid nitrogen. When the drops are frozen and the liquid nitrogen has evaporated they can be stored at -40°C. Long-term storage may be needed for control samples for routine use or to retain a reference preparation of a rare haemoglobin. These methods are generally satisfactory for cellulose acetate and acid gel electrophoresis and for high performance liquid chromatography (HPLC) and capillary electrophoresis.

Haemoglobin electrophoresis

Haemoglobin electrophoresis [5, 6] was previously the most common technique for the initial detection and characterisation of a variant haemoglobin, and is still widely used in many low- and middle-income countries. In most countries, it has now been supplanted in this role by HPLC and capillary electrophoresis but remains useful as a second confirmatory technique. Haemoglobin electrophoresis depends on the principle that when proteins applied to a membrane are exposed to a charge gradient (Fig. 2.2), they separate from each other and can then be visualised by either a protein stain or a haem stain (Fig. 2.3). Haemoglobin electrophoresis can be carried out on filter paper, a cellulose acetate membrane, a starch gel, a citrate agar gel or an agarose gel. Haemoglobin

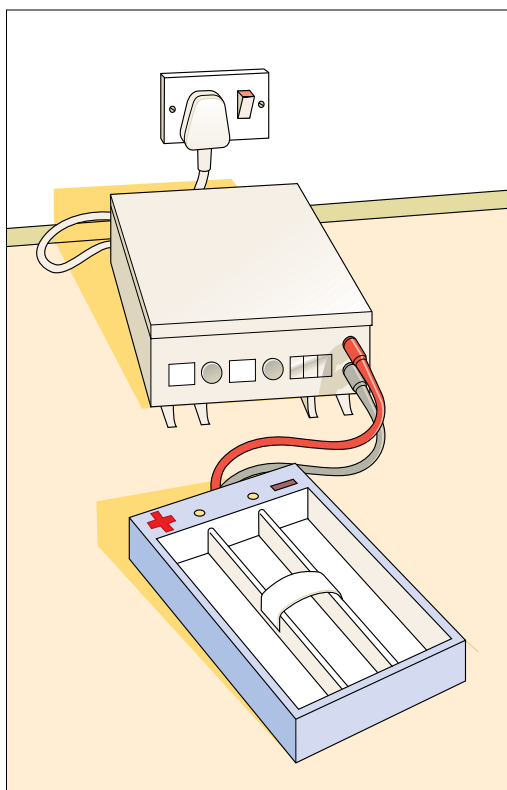


Fig. 2.2 Diagram of apparatus for performing haemoglobin electrophoresis.

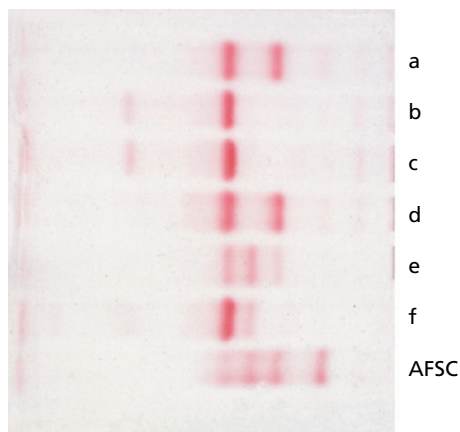


Fig. 2.3 Haemoglobin electrophoresis on cellulose acetate at pH8.3 showing: (a) haemoglobins A and S (sickle cell trait); (b) haemoglobins H and A (haemoglobin H disease); (c) haemoglobins H and A (haemoglobin H disease); (d) haemoglobins A and S (sickle cell trait); (e) haemoglobins A, F and S (sickle cell trait in a baby); (f) haemoglobins A and F (normal baby); (AFSC) control sample containing haemoglobins A, F, S and C.

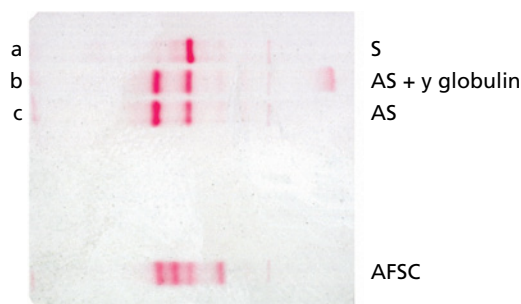


Fig. 2.4 Haemoglobin electrophoresis on cellulose acetate at pH 8.3 showing an abnormal band caused by the presence of a paraprotein: (a) patient with sickle cell anaemia; (b) patient with sickle cell trait and a paraprotein band; (c) patient with sickle cell trait; (AFSC) control sample containing haemoglobins A, F, S and C.

electrophoresis is best performed on lysed packed red cells so that a consistent amount of haemoglobin is applied and so that there are no bands caused by the presence of plasma proteins. If whole blood is used, the presence of a paraprotein or a very high concentration of polyclonal immunoglobulins can lead to a prominent band that can be confused with a variant haemoglobin (Fig. 2.4). If this is suspected, in a laboratory using whole blood for lysate preparation, plasma should be removed and packed red cells should be washed before a new lysate is prepared.

In the body, the presence of a variant β globin chain may lead to two types of abnormal tetramer: one with two variant chains and one with one variant chain and one normal chain. Under the conditions of electrophoresis, tetramers tend to dissociate into dimers; fast-moving dimers reassemble with each other as do slow-moving dimers so that only a single abnormal band representing molecules with two copies of the variant chain is seen. Exceptionally, variant haemoglobins have an abnormally stable tetramer so that hybrid tetramers persist under the conditions of electrophoresis and two variant bands are seen. The situation is more complex in the case of an α chain variant, when there will also be variant haemoglobins A_2 and F with the possibility of hybrid tetramers.

Cellulose acetate electrophoresis at alkaline pH

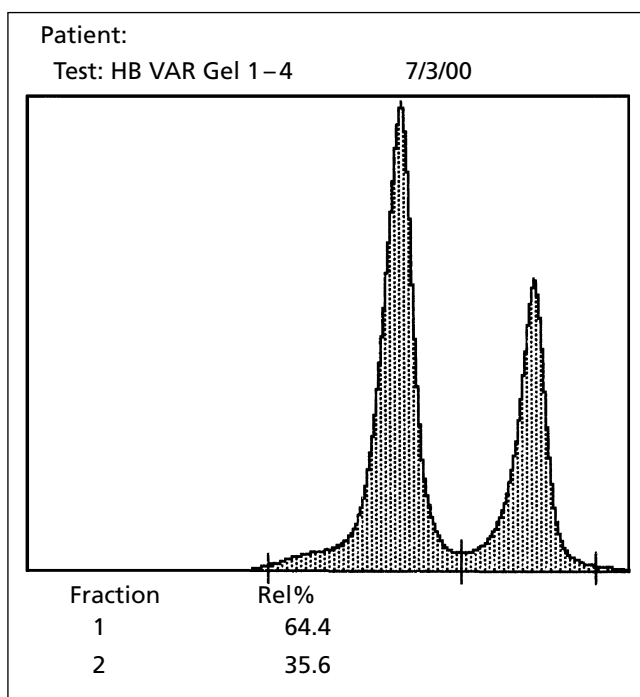
Cellulose acetate electrophoresis at alkaline pH (pH8.2–8.6) permits the detection and provisional identification of haemoglobins A, F, S/G/D/Lepore, C/E/O-Arab, H and a number of less common variant haemoglobins (see Fig. 2.3). Separation is largely but not entirely determined by the electrical charge of the haemoglobin molecule. At this pH, haemoglobin is a negatively charged protein and will move towards the positively charged anode. A control sample containing haemoglobins A, F, S and C should be run with each set of samples. It should be noted that if a protein stain rather than a haem stain is used, carbonic anhydrase will be apparent in addition to haemoglobin bands, moving behind haemoglobin A₂. With good electrophoretic techniques, haemoglobin F levels greater than 2% can be recognised visually. Good techniques also permit a split A₂ band and a significant elevation or reduction of the percentage of haemoglobin A₂ to be recognised visually. Recognition of a split A₂ band is useful in distinguishing α chain variants, such as haemoglobin G-Philadelphia, from β chain variants, such as haemoglobin D-Punjab. A split A₂ band will be present when there is an α or δ chain variant but not when there is a β chain variant. Recognition of a split A₂ band is essential if β thalassaemia trait is to be diagnosed in individuals who also have a δ chain variant. Recognition of a split A₂ band on cellulose acetate electrophoresis requires a fairly heavy application of haemolysate and can be difficult; HPLC (see later) is much more reliable. Recognition of increased haemoglobin F on an electrophoretic strip should be followed by precise quantification whenever this is necessary for diagnosis. A visual estimation of the proportion of haemoglobin A₂ can be used as a supplement to precise measurement by an appropriate technique.

If a variant haemoglobin with the mobility of haemoglobin S is detected, haemoglobin electrophoresis should be followed by a sickle solubility test. If this is negative or if abnormal bands with other mobilities are present, a supplementary

alternative technique (e.g. electrophoresis at acid pH or HPLC) should be used. If bands are very faint or if haemoglobin A appears to be absent (e.g. in a neonatal sample), a supplementary alternative technique should also be used since both HPLC and electrophoresis on agarose gel at acid pH are more sensitive techniques for the detection of a low concentration of a variant or normal haemoglobin. A supplementary alternative procedure is also indicated in patients with a positive sickle solubility test and a single band with the mobility of S, in order to distinguish homozygosity for haemoglobin S from compound heterozygosity for S and β chain D or G variants, haemoglobin Korle Bu and haemoglobin Lepore. Flow charts indicating the sequential application of appropriate tests are given in Chapter 7.

Haemoglobin electrophoresis on cellulose acetate can be used for quantification of normal or variant haemoglobins, either by scanning densitometry (Fig. 2.5) or by elution followed by spectrophotometry. Scanning densitometry requires that the cellulose acetate membrane be rendered transparent by use of a clearing solution, a procedure that some laboratories use routinely, even when scanning is not intended. Scanning densitometry is sufficiently precise to quantify haemoglobins that are present as a large percentage of total haemoglobin. For example, this technique is adequate to determine the percentage of haemoglobin S to permit a distinction between sickle cell trait and sickle cell/ β thalassaemia or to monitor the percentage of haemoglobin S when sickle cell anaemia is being treated by exchange transfusion. Quantification by densitometry can also be used to help distinguish haemoglobin Lepore from other haemoglobins with the same mobility as haemoglobin S; haemoglobin Lepore comprises about 10% of total haemoglobin whereas haemoglobins D and G comprise 25–50%. Quantification of the haemoglobin A₂ percentage by scanning densitometry is not sufficiently precise for the diagnosis of β thalassaemia trait. When cellulose acetate electrophoresis is used for quantification of haemoglobin A₂, elution and spectrometry are required. This is a labour-intensive technique and, when large numbers of

Fig. 2.5 Scanning densitometry of a cellulose acetate electrophoretic strip showing quantification of haemoglobin S in a patient who had had an exchange transfusion for sickle cell anaemia; there is approximately 64% haemoglobin A and approximately 36% haemoglobin S.



samples require testing, HPLC or automated capillary electrophoresis (see later) is preferred. Microcolumn chromatography is now rarely used for this purpose.

Typical mobilities of normal and variant haemoglobins on cellulose acetate electrophoresis at alkaline pH are shown diagrammatically in Fig. 2.6. It should be noted that haemoglobin A₂ has the same electrophoretic mobility as haemoglobins C, E, O-Arab and the S-G-Philadelphia hybrid and it therefore cannot be quantified by cellulose acetate electrophoresis when any of these variant haemoglobins is present. There are subtle differences in the mobility of haemoglobins D-Punjab and Lepore, in comparison with haemoglobin S; they are both slightly anodal (i.e. slightly faster). Nevertheless, various D and G haemoglobins cannot be reliably distinguished from haemoglobin S by use of this technique in isolation and haemoglobin Lepore can be easily distinguished only because it is present in a much lower amount. There are also subtle differences between the mobilities of C and E with haemoglobin C moving slightly more slowly than haemoglobin E (i.e. being

more cathodal) and usually constituting a higher percentage. Nevertheless, a second confirmatory technique is obligatory.

Other characteristics of haemoglobins that have the same mobility as haemoglobin S or haemoglobin C on cellulose acetate electrophoresis are shown in Tables 2.1 and 2.2.

Agarose gel electrophoresis at alkaline pH

Agarose gel is an alternative to cellulose acetate for electrophoresis at alkaline pH (Fig. 2.7). It is somewhat more sensitive for the detection of variant haemoglobins present in small amounts but is more expensive and less convenient than cellulose acetate electrophoresis and has been superseded by other methods.

Citrate agar or agarose gel electrophoresis at acid pH

If a variant haemoglobin is detected by electrophoresis on cellulose acetate or agarose at alkaline pH, it is necessary to confirm its identity by

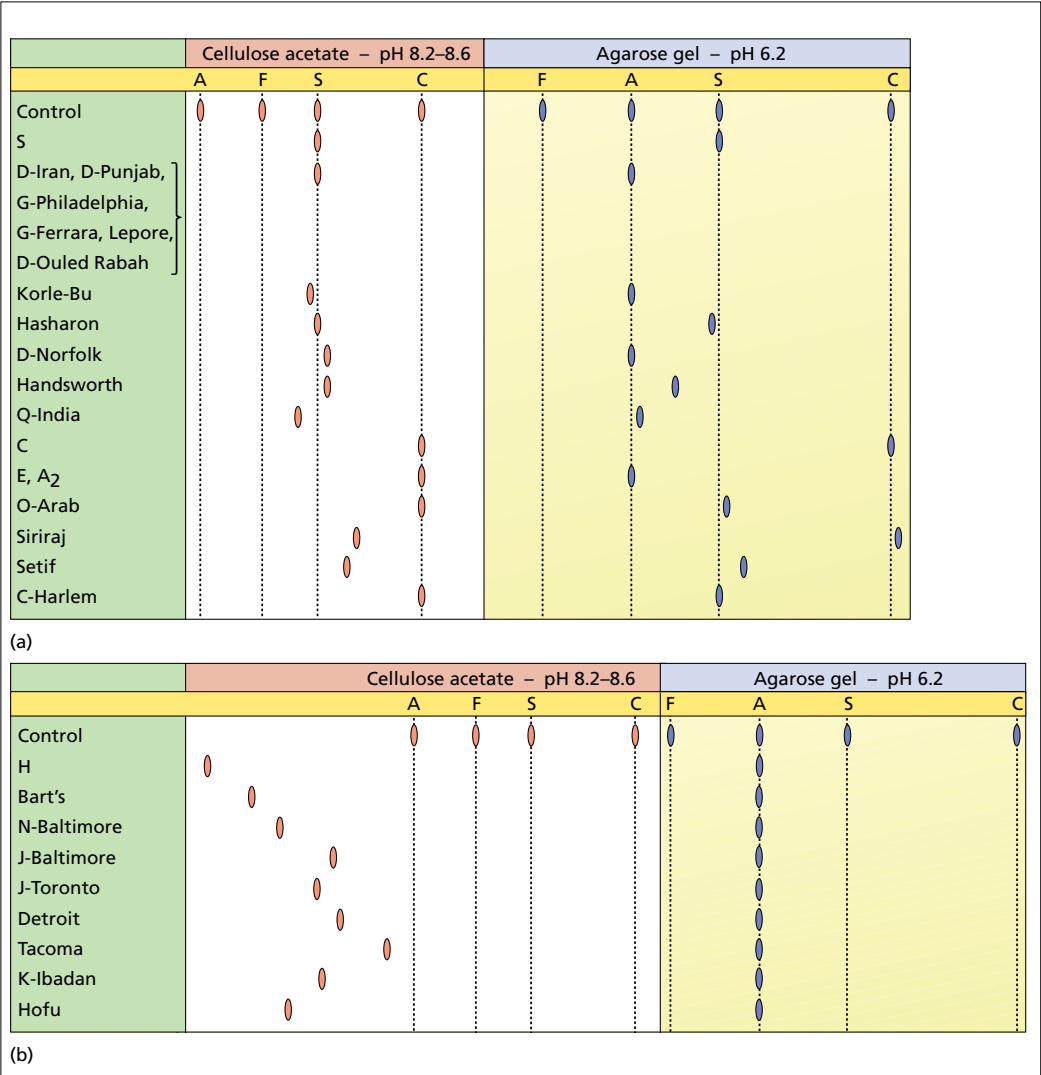


Fig. 2.6 Diagram showing the mobility of normal and variant haemoglobins on cellulose acetate at pH8.2–8.6 in comparison with the mobility on agarose gel at pH6.0–6.2: (a) haemoglobins with mobility close to A, S or C; (b) fast haemoglobins.

Table 2.1 Characteristics of haemoglobin S and some variant haemoglobins with the same mobility as haemoglobin S on cellulose acetate electrophoresis at alkaline pH.

Haemoglobin	Abnormal globin chain	Usual percentage	Mobility on agarose gel at acid pH	HPLC	Usual ethnic origin
S	β	40–45*	S	S window	African ancestry, Arab, Indian
D-Punjab	β	40–45*	With A	D window	Punjabi, Northern European, Greek, Turkish, Yugoslav, African- American, African-Caribbean, Chinese
G-Philadelphia	α	20–25† 25–35† 35–45†	With A	D window‡	African ancestry, Chinese, Italian
Lepore	$\delta\beta$ fusion	7–15	With A	A ₂ window	Greek, Italian, Turkish, Cypriot, Eastern European, English, Spanish, African-Caribbean
Korle Bu	β	40–45	With A§	A ₂ window	West African ancestry
G-Coushatta	β	40–45%	With A	A ₂ window	Native American, Chinese, Korean, Japanese, Thai, Italian, Turkish, Algerian
D-Iran	β	36–45	With A	A ₂ window	Iran, Pakistan, Italy, Jamaica
Zurich	β	22–35	With A	A ₂ window	Swiss, Japanese
Hasharon	α	15–20 (if Jewish) or 30–35 (if Italian)	With S	Between S and C‡	Ashkenazi Jewish, Italians from Ferrara district

HPLC, high performance liquid chromatography.

* Lower if coexisting α thalassaemia trait.

† Depending on the number of normal α genes present.

‡ Variant haemoglobin A₂ also present.

§ Or slightly on the S side of A; if present with S gives a broader band than S alone.

Table 2.2 Characteristics of haemoglobin C and some variant haemoglobins with the same mobility as haemoglobin C on cellulose acetate electrophoresis at alkaline pH.

Haemoglobin	Relevant globin chain	Usual percentage	Mobility on agarose gel at acid pH	HPLC	Usual ethnic origin
A ₂	δ	2–3.5*	With A	A ₂ window	All (normal minor haemoglobin)
C	β	40–45†	C	C window	West African ancestry
E	β	30–35‡	With A	Often A ₂ window	South-east Asian, Bangladesh, Indian (Bengal), Pakistan, Sri Lanka
O-Arab	β	40–45	Slightly on C side of S§	Between S and C windows but closer to C window	Eastern European, African-American, African-Caribbean
C-Harlem	β	40–45†	With S§	Between S and C but closer to C window	West African ancestry
E-Saskatoon	β	35–40	With A	S window	Scottish, Turkish

HPLC, high performance liquid chromatography.

* Compared with 3.5–8% in most β thalassaemia trait.

† Lower if coexisting α thalassaemia trait.

‡ This percentage includes haemoglobin A₂, which has a very similar retention time.

§ O-Arab and C-Harlem are more readily distinguished from each other on citrate agar than on agarose; on citrate agar at acid pH, C-Harlem moves with S and O-Arab is between S and A but closer to A.

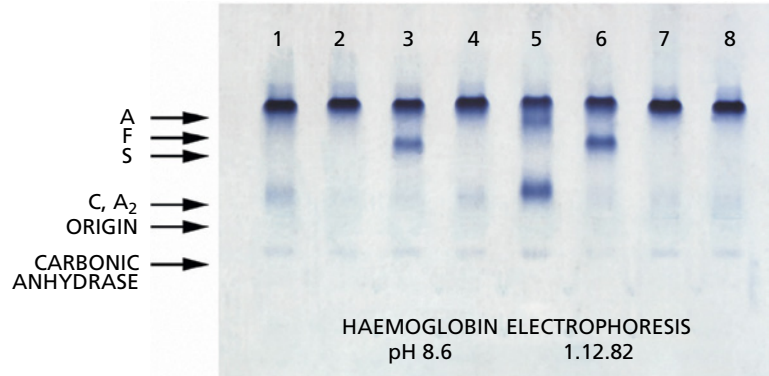


Fig. 2.7 Haemoglobin electrophoresis on agarose gel at alkaline pH (pH 8.6): (1) haemoglobin A and increased A₂ (β thalassaemia trait); (2) haemoglobins A and A₂ (normal); (3) haemoglobin A and S (sickle cell trait); (4) haemoglobins A and A₂ (normal); (5) haemoglobins A, F and C (haemoglobin C trait in a baby); (6) haemoglobin A and S (sickle cell trait); (7) haemoglobins A and A₂ (normal); (8) haemoglobins A and A₂ (normal).

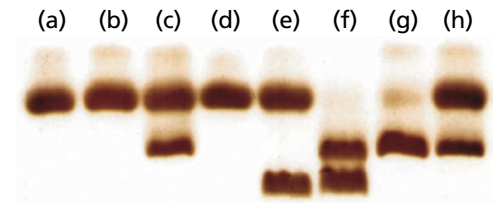


Fig. 2.8 Haemoglobin electrophoresis on citrate agar at pH 6.0–6.2 showing from left to right: (a) normal (haemoglobin A); (b) normal (haemoglobin A); (c) sickle trait (haemoglobins A and S) with co-inheritance of G-Philadelphia; (d) haemoglobins A and J; (e) haemoglobins A and C; (f) haemoglobins S and C; (g) haemoglobin S; (h) haemoglobins A and S. (With thanks to Dr Barbara Wild.)

an alternative technique. When HPLC is not available, this can be electrophoresis on a citrate agar (Fig. 2.8) or, much more commonly, agarose gel (Fig. 2.9) at acid pH (e.g. pH 6.0–6.2). Separated haemoglobins are stained with a haem stain such as o-dianisidine or o-tolidine. With this technique, separation of haemoglobins is dependent not only on their electrical charge but also on their interaction with various components in the agar or agarose. Agar contains both agarose and agarpectin, a sulphated polysaccharide [7]. Agarose polymerises and is immobile but agarpectin is able to complex with some of the amino acids of haemoglobin and the haemoglobin–agarpectin complex then migrates towards the anode while any non-complexed haemoglobin is carried towards

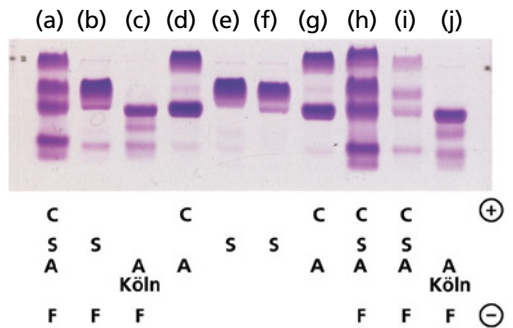


Fig. 2.9 Haemoglobin electrophoresis on agarose gel at pH 6.0–6.2 (with variant haemoglobins listed from below upwards) showing from left to right: (a) control sample with haemoglobins F, A, S and C; (b) F and S (sickle cell anaemia); (c) haemoglobins F, Köln and A (heterozygosity for haemoglobin Köln); (d) haemoglobins A and C (haemoglobin C trait); (e) haemoglobin S (sickle cell anaemia); (f) haemoglobin S (sickle cell anaemia); (g) haemoglobins A and C (haemoglobin C trait); (h) control sample with haemoglobins F, A, S and C; (i) control sample with haemoglobins F, A, S and C; (j) haemoglobins F, Köln and A (heterozygosity for haemoglobin Köln).

the cathode by endo-osmotic flow [7]. There are some differences in the relative mobilities of variant haemoglobins between agarose gel (see Fig. 2.6) and citrate agar (Fig. 2.10). Both techniques distinguish S from D/G but do not distinguish between most types of D and G. Electrophoresis at acid pH will distinguish haemoglobin C from E, C-Harlem and O-Arab

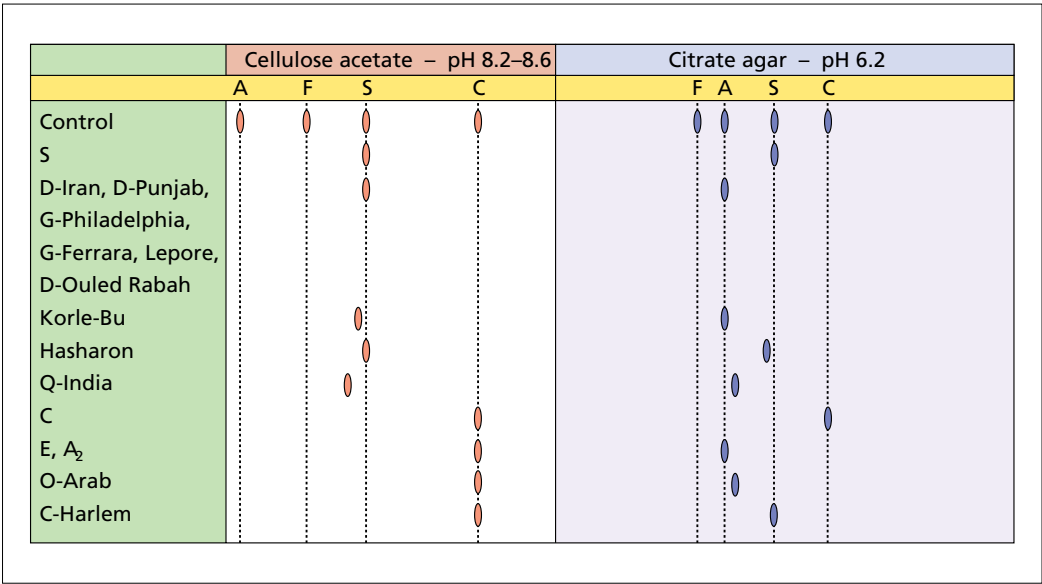


Fig. 2.10 Diagram showing the mobility of normal and variant haemoglobins on cellulose acetate at pH 8.2–8.6 in comparison with the mobility of citrate agar gel at pH 6.0–6.2; the fast haemoglobins shown in Fig. 2.6(b) have the same mobility as haemoglobin A on citrate agar at acid pH.

and will help to distinguish the latter three variant haemoglobins from each other. Acid agar and agarose gel electrophoresis do not resolve haemoglobin A₂ from haemoglobin A. Electrophoresis at acid pH may be indicated in the investigation of suspected high affinity haemoglobins even when electrophoresis at alkaline pH is normal, since some high affinity haemoglobins have abnormal mobility only at acid pH.

Capillary electrophoresis

In this technique, also known as capillary zone electrophoresis, the procedure is carried out in capillary tubes, permitting higher voltages to be used and shortening the run time. It is dependent on ion migration in an alkaline buffer and electro-osmotic flow to separate haemoglobins, which are quantified as they flow past a detector. The position of a haemoglobin in relation to haemoglobin A is used to determine into which 'zone' it falls. Identification of the zone requires the presence in the sample of both haemoglobins A and A₂ or, alternatively, F and A₂. An electropherogram is produced (Figs 2.11 and 2.12). There is a drop-down menu indicating which

normal and variant haemoglobins are found in each zone. This method has the further advantage of small sample size, which makes it suitable for neonatal screening programmes (Fig. 2.13). Fractions due to post-translational modification of haemoglobins A, S, C and other haemoglobins are not separated from the unmodified haemoglobin. This simplifies interpretation but does mean that an incidental diagnosis of diabetes mellitus, something which is not infrequent with HPLC performed for investigating a haemoglobinopathy (see later), will not be made. Degraded haemoglobins, as with an aged sample, may give an extra peak; for example, degraded S moves with F [8]. Capillary electrophoresis optimised for measurement of haemoglobin A_{1c} occasionally detects a variant haemoglobin that is not detected by the standard haemoglobinopathy method and sometimes also not by HPLC [8]. Some variants that appear identical on a capillary electrophoresis haemoglobinopathy programme, for example haemoglobin Q-India and haemoglobin Q-Iran, can be distinguished on a haemoglobin A_{1c} programme. Capillary electrophoresis can separate haemoglobin A₂ from haemoglobin E but not always from

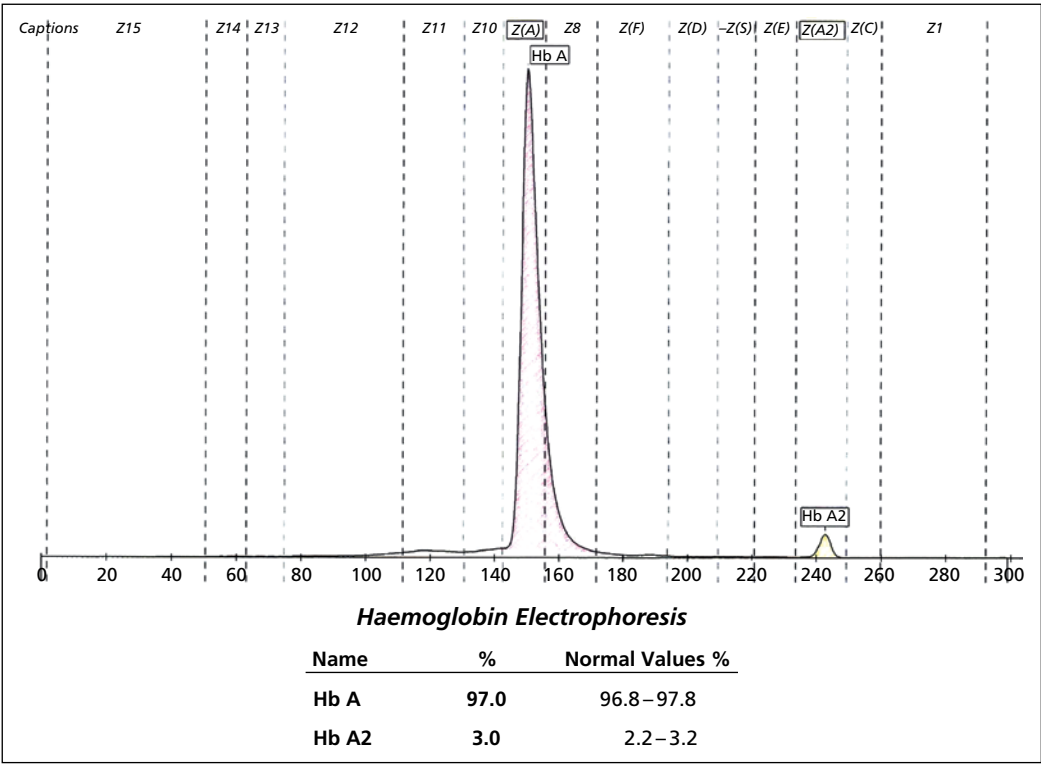


Fig. 2.11 Capillary electrophoresis electropherogram, Sebia Capillarys 3, showing haemoglobins A and A₂.

haemoglobin C so that, for some samples, C and A₂ are measured together [9, 10]. Separation of haemoglobin A₂ from haemoglobin Lepore is also possible [11]. Haemoglobin O-Arab is superimposed on haemoglobin A₂. Haemoglobin F may be overestimated in patients with diabetes mellitus because of overlapping of haemoglobin F and glycated haemoglobin A [12]. In samples containing haemoglobin D or S, estimation of haemoglobin A₂ by capillary electrophoresis can be more accurate than estimation by HPLC. However, haemoglobin A₂ may be overestimated when there are overlapping peaks that are not fully integrated [8]. Haemoglobin F cannot be quantified accurately below 0.3% but this is not of any consequence. The method is suitable for the detection of haemoglobin Constant Spring and is more sensitive than HPLC [13]. However, it should be noted that this variant may become undetectable if a specimen is stored for more

than seven days [14]. Haemoglobin Constant Spring can be identified in the presence of β thalassaemia trait or haemoglobin E trait [15]. Haemoglobin Bart's and haemoglobin H (Fig. 2.14) can also be suspected [16].

In two studies using Sebia instruments, the haemoglobin A₂ reference range was 2.1–3.2 [17] and 2.25–3.05 [10] respectively and for haemoglobin F was <0.8% [17] and <0.6% respectively [10].

An online atlas is available. <https://extranet.sebia.com/user>.

High performance liquid chromatography

Cation-exchange high performance liquid chromatography (CE-HPLC or HPLC) is a process in which a mixture of molecules (such as normal and variant haemoglobins) with a net positive charge is separated into its components by their

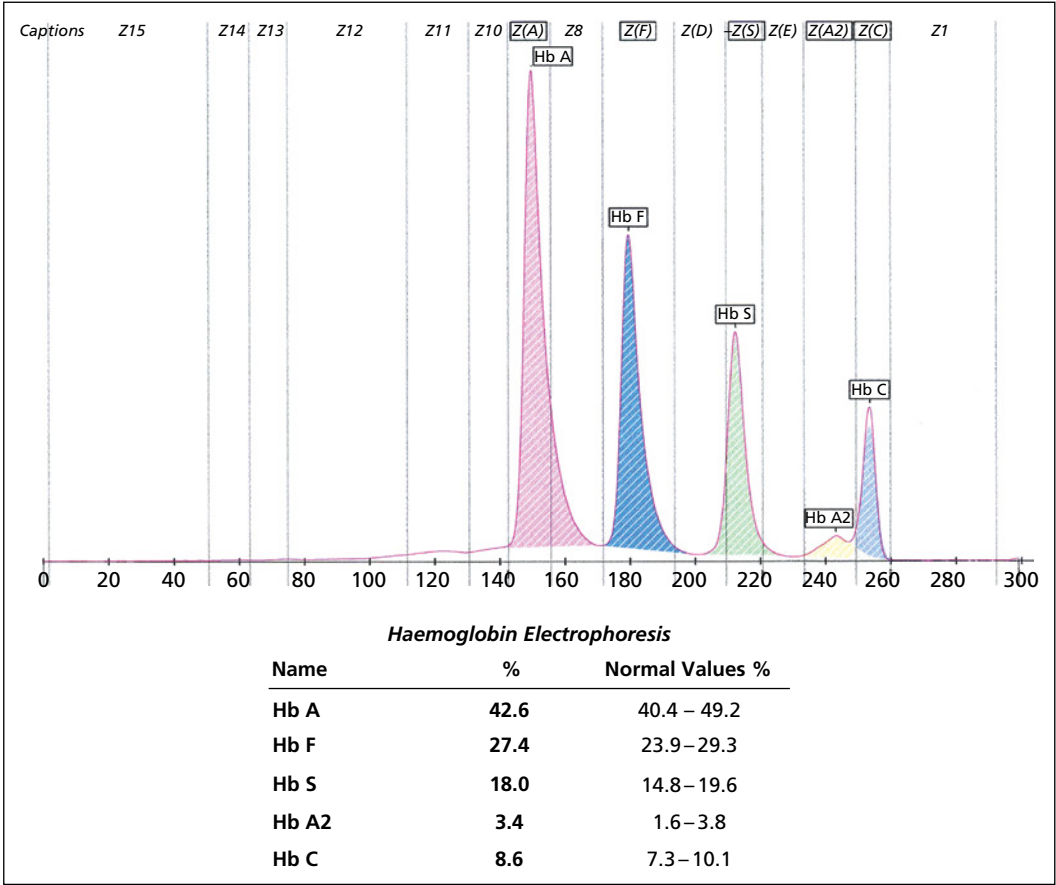


Fig. 2.12 Capillary electrophoresis electropherogram, Sebia Capillarys 3, of an artificial mixture showing the relative positions of haemoglobins A, F, S, A₂ and C.

adsorption onto a negatively charged stationary phase in a chromatography column, followed by their elution by a mobile phase. The mobile phase is a liquid with an increasing concentration of cations flowing through the column; the cations in the mobile phase compete with the adsorbed proteins for the anionic binding sites. Thus the adsorbed positively charged haemoglobin molecules are eluted from the column into the liquid phase at a rate related to their affinity for the stationary phase. When separated in this way, they can be detected optically in the eluate, provisionally identified by their retention time and quantitated by computing the area under the corresponding peak in the elution profile. There is some correlation between HPLC retention times and mobility on

cellulose acetate electrophoresis at alkaline pH. The more positively charged haemoglobins (e.g. haemoglobins S and C) have a longer retention time, correlating with a slower mobility on cellulose acetate at alkaline pH.

The application of HPLC to the identification of variant haemoglobins depends on the fact that for each normal or variant haemoglobin on a specified system, there is a characteristic period of time, referred to as the retention time, before the haemoglobin appears in the eluate. Many variant haemoglobins can be separated from each other although there are some that overlap with others. Some haemoglobins can be resolved by HPLC that are not resolved by cellulose acetate electrophoresis at alkaline pH. For example, haemoglobins D-Punjab/Los

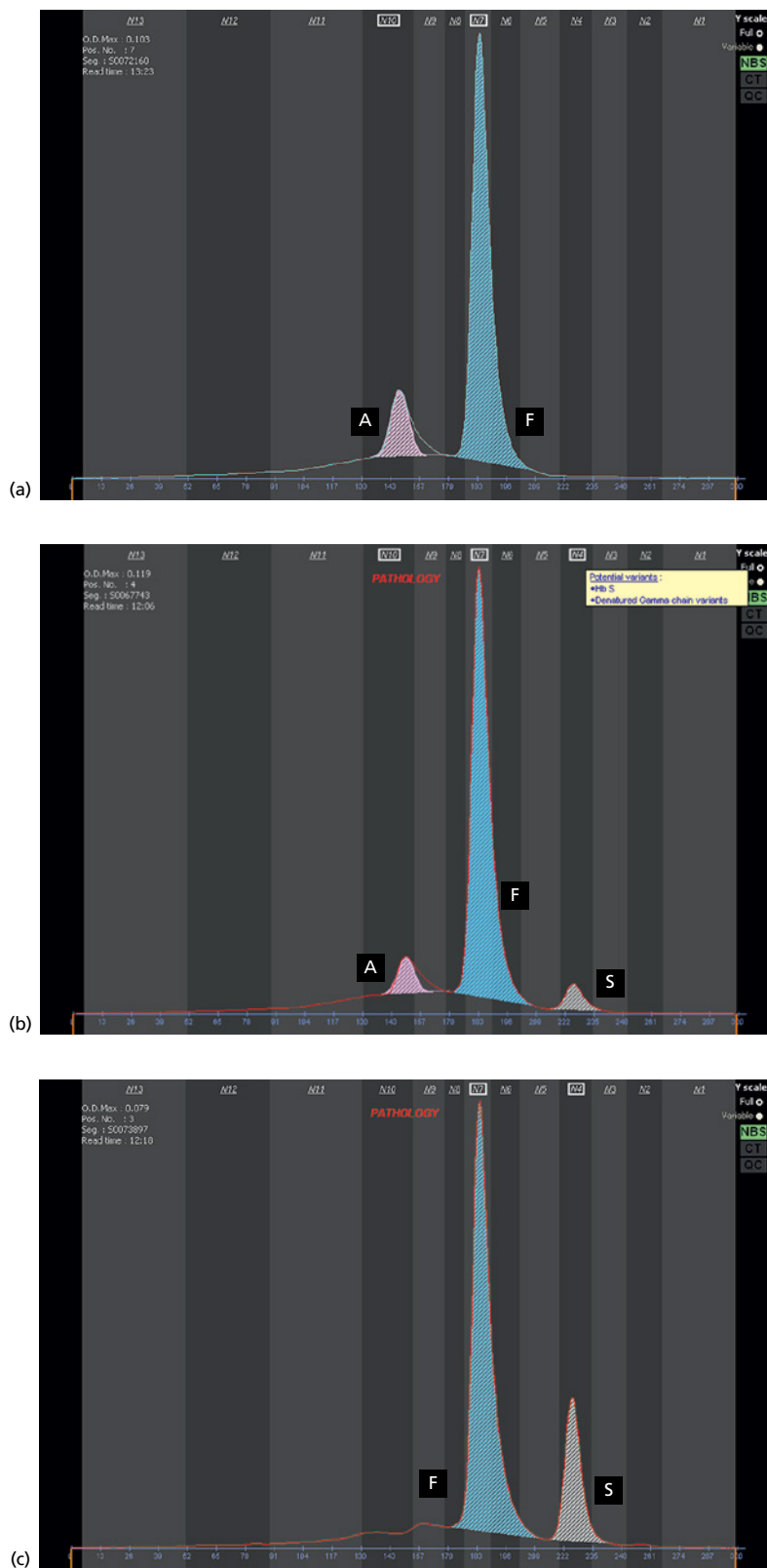


Fig. 2.13 Annotated screen shots showing Sebia Capillars 2 electropherograms of neonatal screening samples: (a) normal baby; (b) sickle cell trait; (c) sickle cell anaemia; (d) haemoglobin C trait; (e) sickle cell/haemoglobin C disease; (f) haemoglobin D trait; (g) haemoglobin E trait. (With thanks to Sarah Brown.) (Continued on pp. 45–46.)

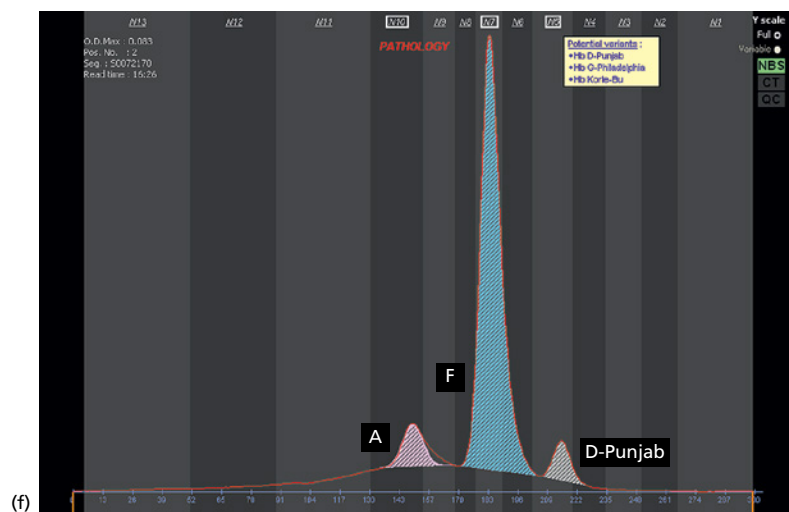
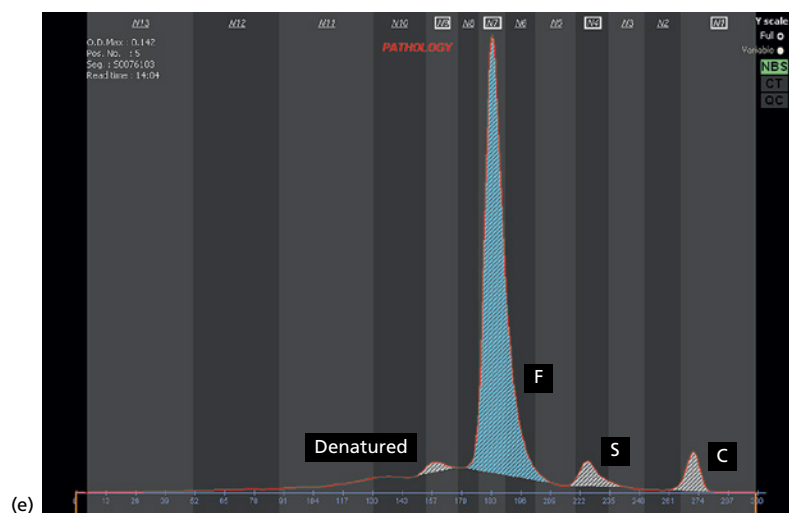
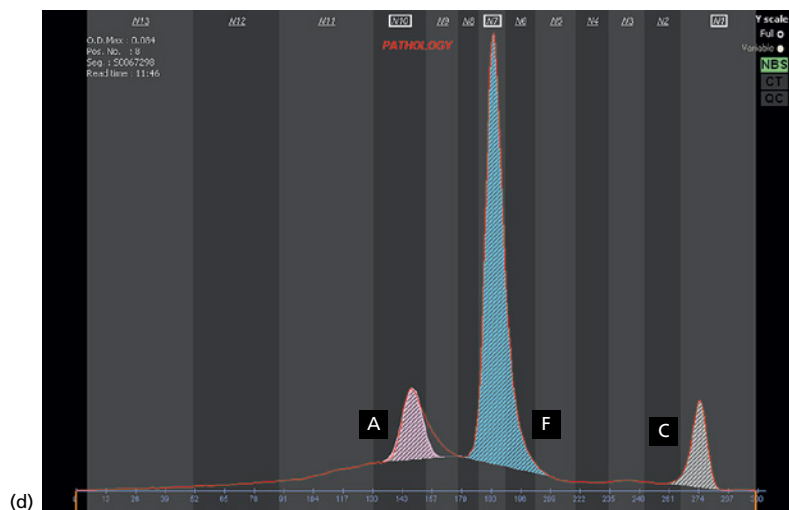


Fig. 2.13 Continued.

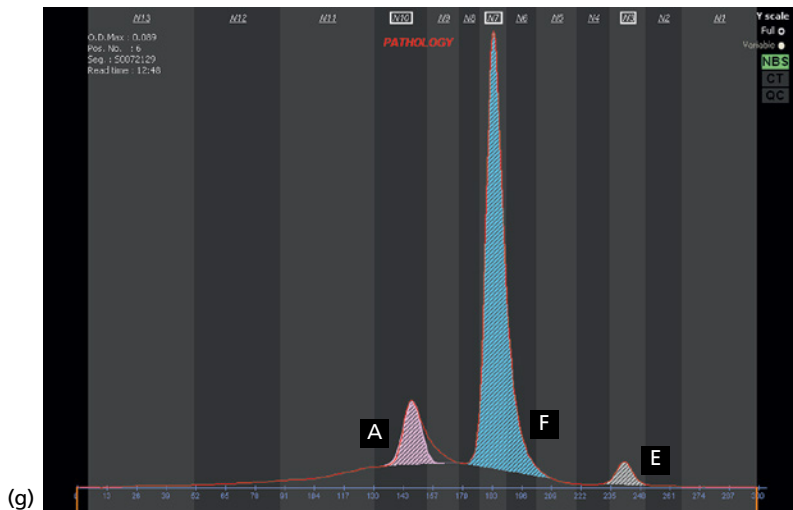


Fig. 2.13 Continued.

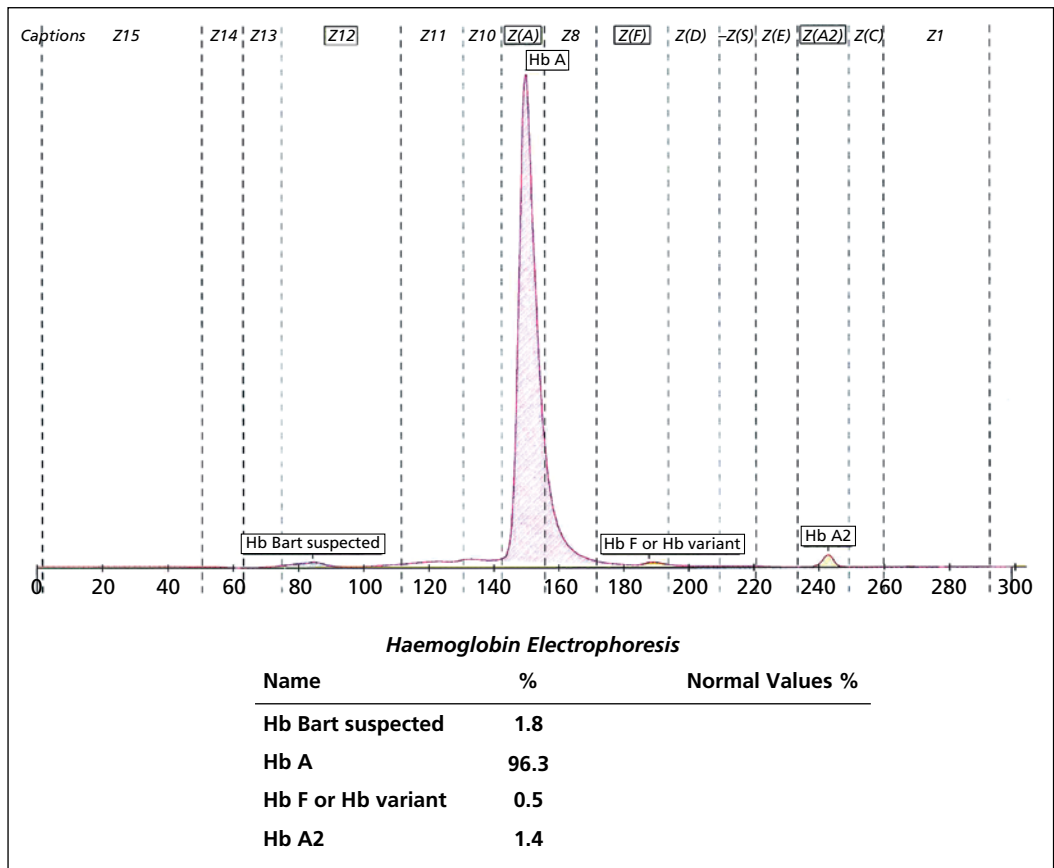


Fig. 2.14 Capillary electrophoresis electropherogram, Sebia Capillarys 3, from a patient with haemoglobin H disease showing haemoglobin H ('Hb Bart suspected'), haemoglobin A, haemoglobin F and haemoglobin A₂. The nature of the haemoglobin H was confirmed by high performance liquid chromatography (HPLC) and by detection of haemoglobin H inclusions. Note that haemoglobin A₂ is reduced, as expected in haemoglobin H disease. The genotype was $\alpha^{37}/-\alpha^{FIL}$.

Angeles and G-Philadelphia can be resolved from haemoglobin S and from each other. A slower rate of flow of buffer improves the separation of different haemoglobins with a similar retention time but increases the time taken to process each sample. The temperature of operation must be carefully controlled to ensure consistent retention times. Haemoglobins eluted from the column are represented graphically and automatically quantified by spectroscopy; the retention time is stated in relation to that expected for a known normal or abnormal haemoglobin (usually in relation to haemoglobin A, F, S, C or D).

Automated HPLC instruments for investigation of haemoglobin currently in use are high precision instruments, which are moderately rapid; they use specially designed microbore columns, high precision gradient-forming liquid pumps and optical detectors. There is computer control and data handling and sometimes computer/intranet-assisted interpretation.

High performance liquid chromatography can be used not only for the detection, provisional identification and quantification of variant haemoglobins but also for the quantification of haemoglobins A, A₂ and F. Control materials for monitoring the precision of measurements of haemoglobins F and A₂ are commercially available. HPLC has the following advantages over haemoglobin electrophoresis:

- it is less labour intensive;
- a very small sample is adequate;
- quantification of normal and variant haemoglobins is available for each sample;
- since haemoglobin A₂ is quantified, β thalassaemia trait can be diagnosed in a single procedure;
- a larger range of variant haemoglobins can be provisionally identified;
- many haemoglobin A₂ variants can be detected easily, thus facilitating the differentiation of α and β chain variants (even those with identical retention times) and making the diagnosis of β thalassaemia trait when a δ chain variant is present more accurate.

The main disadvantage is the higher capital and reagent costs, and the need for regular servicing and maintenance of these complex

machines. However, if the greater labour costs of electrophoresis are considered, then in developed countries with high wages the overall costs are comparable [18] and the possibility of rapid processing of a large number of samples is a considerable advantage.

Considerable skill and experience are needed in interpreting the results of HPLC since the data produced are quite complex. Glycosylated variant haemoglobins have a different elution time from the non-glycosylated forms and acetylated haemoglobins from the non-acetylated forms (haemoglobin F is partially acetylated). In addition, a variant haemoglobin may have the same retention time as either a normal haemoglobin or another variant. For example, haemoglobin E, haemoglobin Korle Bu, haemoglobin Fort Worth and haemoglobin Lepore can overlap with haemoglobin A₂, and haemoglobin A₂ can be falsely elevated in the presence of haemoglobin S and falsely reduced in the presence of haemoglobin D-Punjab.

Measurement of haemoglobin A_{1c}, haemoglobin A in which the N-terminal valine is irreversibly glycated, is used for predicting a diagnosis of diabetes mellitus and for monitoring the adequacy of control of this condition, the rate of glycation and therefore the percentage being higher when blood glucose is often elevated. HPLC is the usual method for quantification but it can be inaccurate in the presence of a variant haemoglobin for the following reasons: (i) the variant haemoglobin may have the same retention time as haemoglobin A_{1c} or the two peaks may overlap, leading to serious overestimation and usually an unrealistic result; (ii) post-translationally modified variant haemoglobin may coincide with or overlap with haemoglobin A_{1c}, leading to overestimation but without making the result unrealistic; (iii) if the haemoglobin A_{1c} is expressed as a percentage of total haemoglobin, ignoring the presence of the variant (and its glycated fraction) the percentage will be underestimated; (iv) the amino acid change present in the variant haemoglobin may alter the rate of glycation of the N-terminal valine. Alternative methods such as affinity chromatography and immunoassay can be used but they also can fail; for example, haemoglobin

Raleigh undergoes post-translational acetylation and the acetylated adduct cannot be glycated [19]. Haemoglobin A_{1c} may be increased significantly (e.g. by approximately 2% or 23 mmol/mol) by iron deficiency, although conflicting results have also been published [20]. Information relating to specific instruments and common variant haemoglobins is available from the NGSP website (<http://ngsp.org>) [21].

Haemoglobinopathy investigations by HPLC not infrequently lead to the diagnosis of previously unsuspected diabetes mellitus when an increased glycated fraction is demonstrated (Fig. 2.15) [22].

Glycated fractions can lead to invalid or misleading results in other ways. A clinically meaningless elevation of glycated haemoglobin can occur in a patient who has been transfused as the red cells will have been exposed to a high glucose concentration during storage. Glycated haemoglobin S may have a retention time the same as, or very similar to, that of haemoglobin A so that patients with sickle cell anaemia may be thought to have a small amount of haemoglobin A (Fig. 2.16). In patients with sickle cell trait, the haemoglobin S percentage was underestimated by 7.4% in one study as a result of the closeness of glycated haemoglobin S to haemoglobin A₀ [23]. With some instruments and programmes, the haemoglobin F can merge with the peak resulting from glycated haemoglobin A and may not be detected when it is 0.6% or less [24]. Conversely, an elevated percentage of glycated haemoglobin can lead to a factitious elevation of haemoglobin F [24]. The percentage of glycated haemoglobin A is noticeably reduced in patients with a shortened red cell life span, e.g. hereditary spherocytosis and haemoglobin H disease, due to the preferential loss of older red cells containing more glycated haemoglobin.

Certain artefacts need to be recognised; for example, increased bilirubin in the plasma can lead to a sharp peak in the same general area as haemoglobin H, haemoglobin Bart's and acetylated haemoglobin F (Fig. 2.17). An early small artefactual peak represents the effect of injection of the sample into the column.

The haemoglobins that can be distinguished from each other vary somewhat between different instruments and reagent systems. However, all systems permit the provisional identification of many more variant haemoglobins than can be distinguished by electrophoresis. Haemoglobins A, A₂, F, S, C, O-Arab, D-Punjab and G-Philadelphia can be separated from each other. With some instruments (e.g. Bio-Rad Variant II and Tosoh G8), haemoglobin E overlaps with haemoglobin A₂, whereas with other instruments (e.g. Agilent 1100 and Trinity Ultra2) the two peaks are distinct [25]. A broad peak on HPLC may indicate the presence of hybrid molecules that have the normal and the variant β chain in the same molecule [26]. The nature of any variant haemoglobin detected by HPLC should be confirmed by an alternative technique.

Some HPLC instruments have dual kits and protocols that permit both haemoglobin A_{1c} and haemoglobin A₂ to be quantified accurately [27, 28]. They also can measure haemoglobin A₂ accurately in the presence of haemoglobin S [27]. However, some variants that separate from haemoglobin A with other instruments may fail to separate with a dual kit [28].

Evaluations of a number of the automated HPLC systems that are now commercially available have been published [24, 29–32].

Typical elution patterns of normal and variant haemoglobins are shown in Figs 2.18–2.21 and some of the variants that can have retention times overlapping with those of haemoglobin A₂ and haemoglobin S are shown in Table 2.3 [30, 33–37]. It should be noted that the 'window' of retention times allocated to a normal or a variant haemoglobin by a manufacturer may be considerably wider than the range of retention times actually observed with that haemoglobin. It is therefore necessary to consider the actual retention time as well as the window in which it falls in making a provisional identification.

In one study using a Bio-Rad Variant II instrument, reference ranges were 2.34–3.2 for haemoglobin A₂ and <0.9% for haemoglobin F [10].

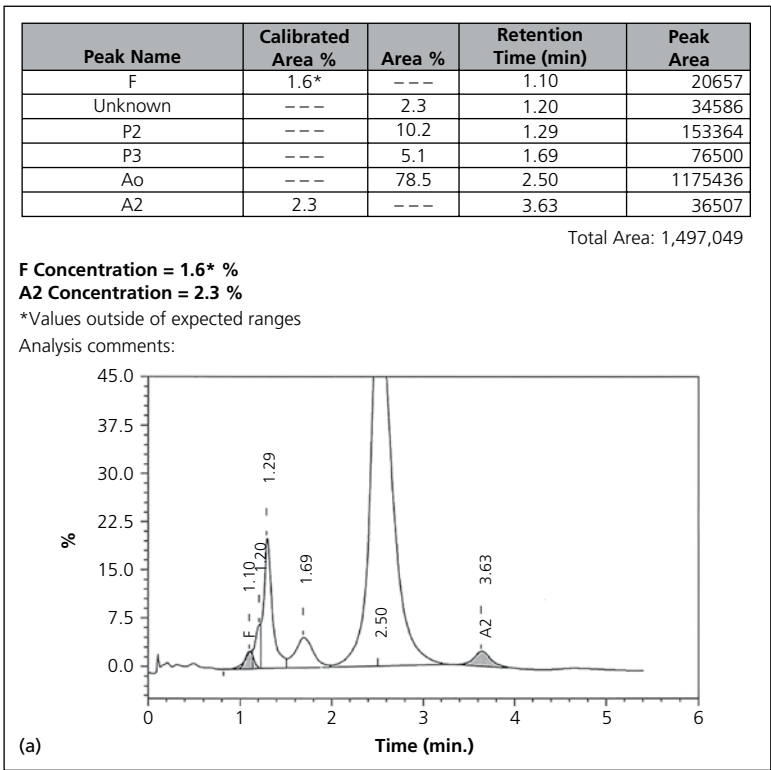
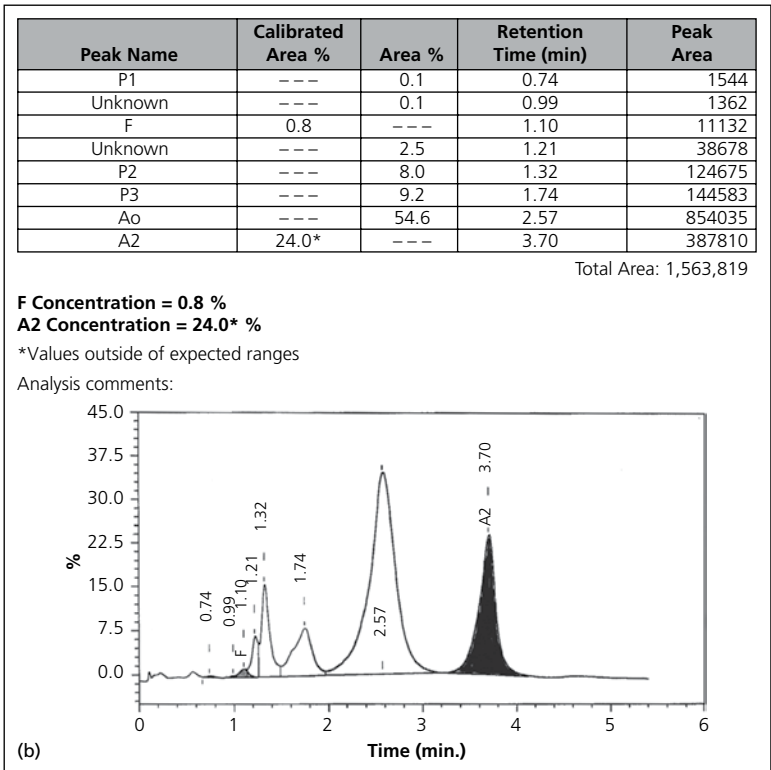


Fig. 2.15 HPLC chromatogram (Bio-Rad Variant II) showing increased haemoglobin A_{1c} (P2) in two patients with diabetes mellitus detected during haemoglobinopathy investigations: (a) in a patient with no variant haemoglobin; (b) in a patient with haemoglobin E trait. A glycated fraction of more than 6% is usually indicative of diabetes. Note that the level of 8% in the patient with E trait will be an underestimate of the total glycated fraction as glycated haemoglobin E is not included.



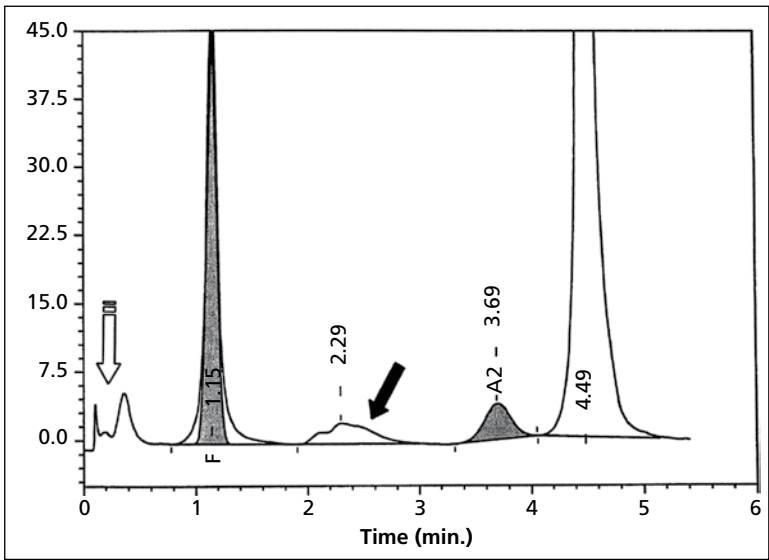


Fig. 2.16 HPLC chromatogram (Bio-Rad Variant II) from a patient with sickle cell anaemia showing glycosylated haemoglobin S with the same retention time as haemoglobin A (black arrow) and haemoglobin F, including the acetylated form (white arrow).

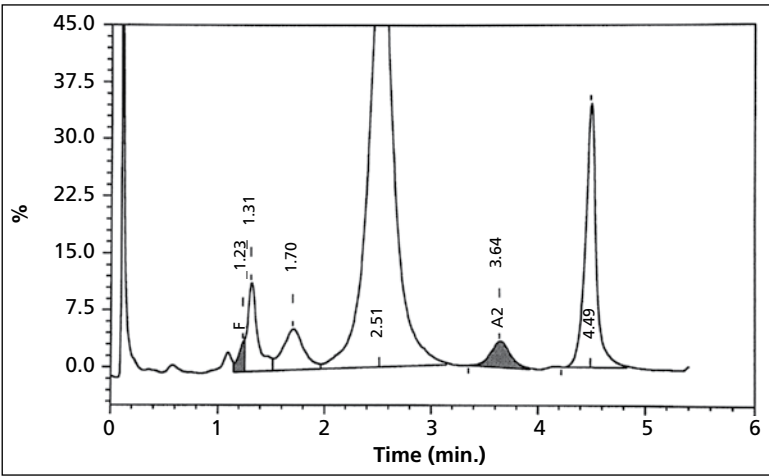


Fig. 2.17 HPLC chromatogram (Bio-Rad Variant II) from a patient with a bilirubin of 970 $\mu\text{mol/l}$ showing a peak in the same region as haemoglobin Bart's; from left to right the peaks are bilirubin, haemoglobin F, altered haemoglobin A (two peaks) and haemoglobins A₁, A₂ and S.

Isoelectric focusing

Isoelectric focusing (IEF) relies on the fact that the net charge of a protein depends on the pH of the surrounding solution. At a low pH the carboxylic acid groups of proteins are generally

uncharged and their N-containing basic groups are fully charged (NH_3^+), giving a net positive charge. At high pH the converse occurs; the carboxylic acid groups are negatively charged (COO^-) and the basic groups are uncharged, giving a net negative charge. In IEF, various

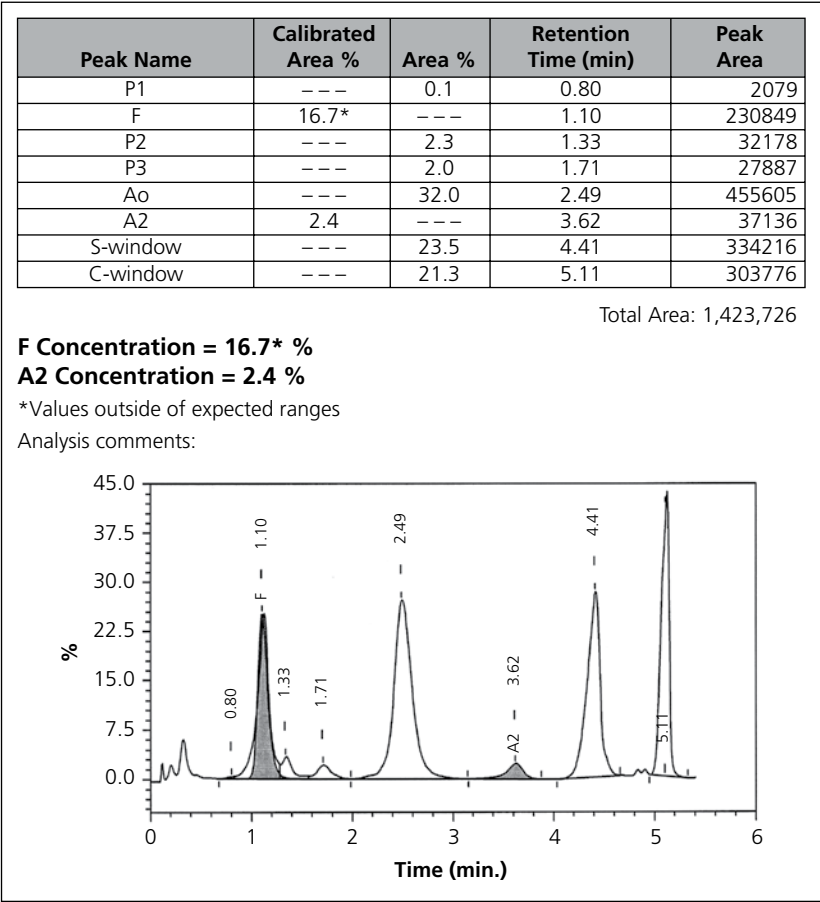


Fig. 2.18 Artificial mixture showing the retention times of normal and important variant haemoglobins on HPLC (Bio-Rad Variant II). From left to right the peaks are acetylated F (complex peak that is not integrated), F₀ (shaded), P2 (glycated A), P3 (other post-translationally modified A), A₀, A₂ (shaded), S and C.

haemoglobins are separated in a gel with a pH gradient (e.g. an agarose gel) according to their isoelectric points (pI), which is the point at which they have no net charge. Commercially available prepared plates of polyacrylamide or cellulose acetate contain carrier amphoteric molecules of various pIs, establishing a pH gradient across the plate. When a haemolysate is applied to the prepared plate in a strong electric field the haemoglobin molecules migrate through the plate until they reach the point at which the pH corresponds to the pI of the haemoglobin. Since the haemoglobin molecule then has no net charge it remains at that point. The various haemoglobin bands are stained (Fig. 2.22) and can be quantified by densitometry

(Fig. 2.23). Densitometric traces can be superimposed on traces of known variants to aid in their identification.

Bands on IEF are sharper than those obtained with cellulose acetate electrophoresis. In addition, some haemoglobins that cannot be distinguished from each other by electrophoresis can be separated by IEF. For example, some D and G variants (such as D-Punjab/Los Angeles and G-Philadelphia) can be separated from haemoglobin S and from each other (Fig. 2.24) [38]. Haemoglobins that can be distinguished from each other by IEF differ between different instrument/reagent systems. Although quantification by densitometry is possible, precision at low concentrations is poor and this method is therefore

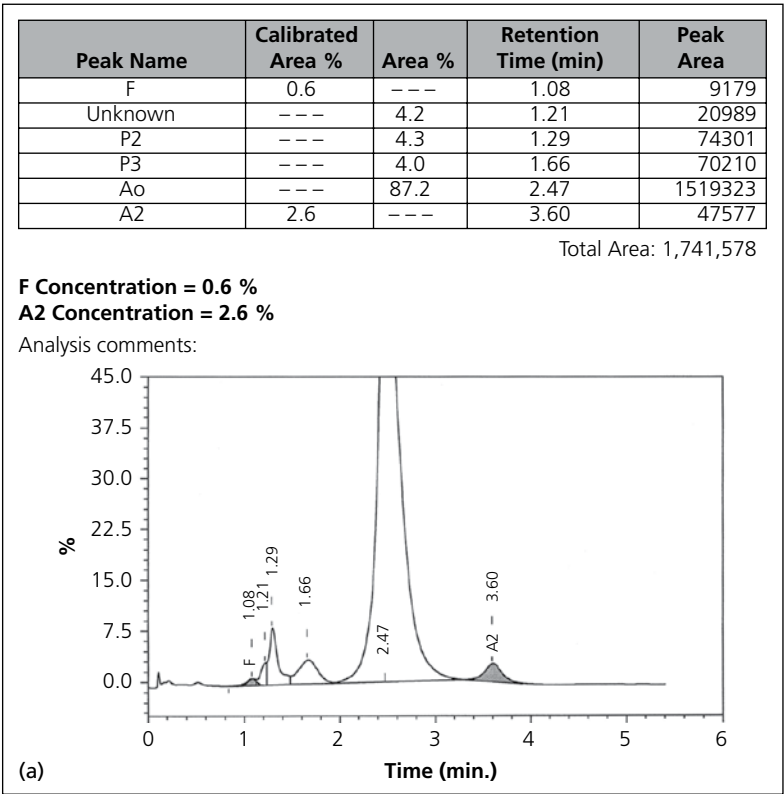


Fig. 2.19 Typical elution patterns on HPLC with the Bio-Rad Variant II system for normal samples and for common or clinically important variant haemoglobins; in each specimen, peaks in the F and A₂ windows are generally crosshatched, P2 represents glycated haemoglobin A, P3 other post-translationally modified A and A₀ unmodified A; the small early peak (far left) is an injection artefact and is ignored in the descriptions that follow: (a) normal adult; (b) normal neonate, peaks from left to right are acetylated haemoglobin F (a complex of peaks), F₀ and A₀ (19.5%); (c) premature neonate, peaks from left to right are acetylated haemoglobin F, F₀ and A₀ (9.1%); (d) sickle cell trait, peaks from left to right are F, P2, P3, A₀ (with the irregularity on the upward slope being caused by glycated S), A₂ (including post-translationally modified S) and S; (e) sickle cell anaemia, peaks from left to right are acetylated F, F₀, glycated S ('unknown' and in A₀ window), A₂ and S; (f) haemoglobin C trait, peaks from left to right are F, P2, P3, A₀, A₂, glycated C (in S window) and C (with a shoulder on the upwards slope representing other post-translationally modified C); (g) haemoglobin C homozygosity, peaks from left to right are F, a low peak in the A₀ window that probably represents carry-over from the preceding specimen, glycated C (in S window) and C (with its shoulder of other post-translationally modified C); (h) sickle cell/haemoglobin C compound heterozygosity, peaks from left to right are glycosylated S (in A₀ window), A₂ (including other post-translationally modified S), S plus glycated C and C (with its shoulder of other post-translationally modified haemoglobin C); (i) haemoglobin E trait, peaks from left to right are F, P2, P3, A₀ and E plus A₂; (j) haemoglobin E homozygosity, peaks from left to right are F, P3 (in this instance, glycated E), other post-translationally modified E (in A₀ window) and E plus A₂; (k) haemoglobin Lepore trait, peaks from left to right are small irregular peaks of acetylated F, F₀, P2, P3, A₀ and Lepore plus A₂; (l) haemoglobin D-Punjab trait, peaks from left to right are F, P2, P3, unidentified (probably glycated D), A₀, A₂ and D-Punjab; (m) haemoglobin G-Philadelphia trait, peaks from left to right are F, P2, P3, A₀ (with a shoulder that is likely to represent glycated G-Philadelphia), A₂, G-Philadelphia and G₂ (an A₂ variant with a G-Philadelphia α chain); (n) heterozygosity for both haemoglobin G-Philadelphia and haemoglobin C, peaks from left to right are F, P2, P3, A₀, A₂, G-Philadelphia, glycated C (in S window), C and C-G-Philadelphia hybrid; (o) haemoglobin O-Arab trait, the peaks from left to right are F, P2, P3, A₀, A₂, glycated O-Arab, other post-translationally modified O-Arab and unmodified O-Arab; (p) haemoglobin S/haemoglobin O-Arab compound heterozygosity, the peaks from left to right are acetylated F, F₀, a peak in the A₀ window representing glycated haemoglobin S, A₂, glycated O-Arab, S, other post-translationally modified O-Arab and unmodified O-Arab. (Continued on pp. 53–60.)

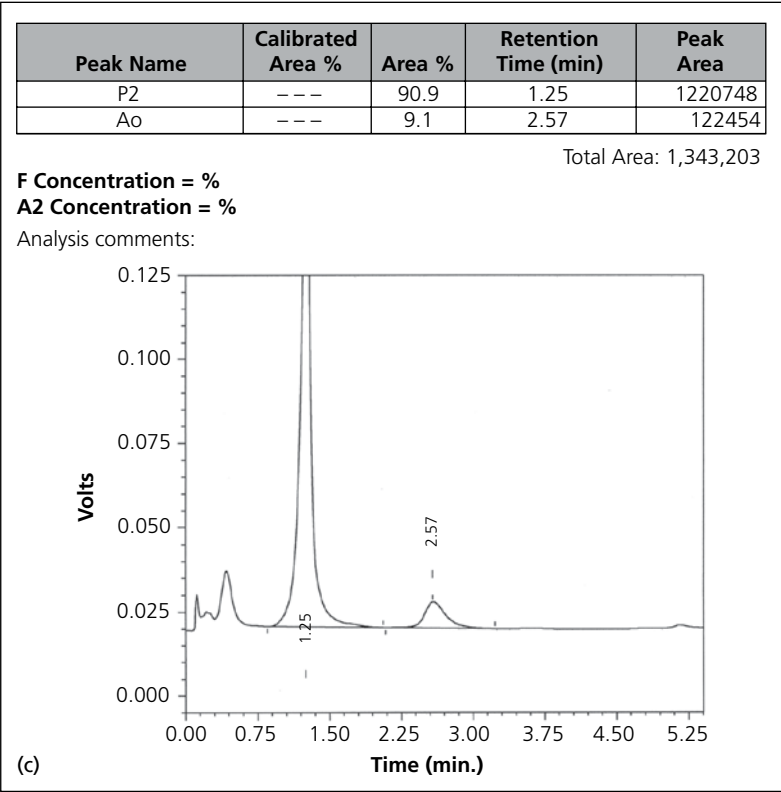
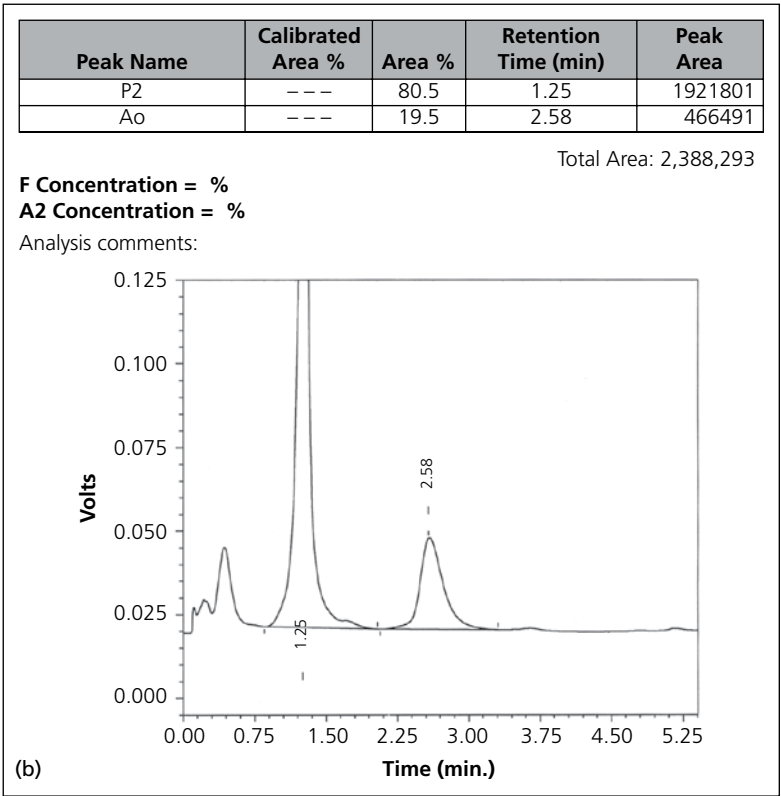


Fig. 2.19 Continued.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.00	1048
F	0.1*	---	1.06	2061
Unknown	---	0.2	1.15	3080
Unknown	---	0.9	1.26	16287
P2	---	4.1	1.33	73824
P3	---	3.4	1.71	62032
Ao	---	52.7	2.57	952857
A2	3.8*	---	3.66	67933
S-window	---	34.8	4.50	630014

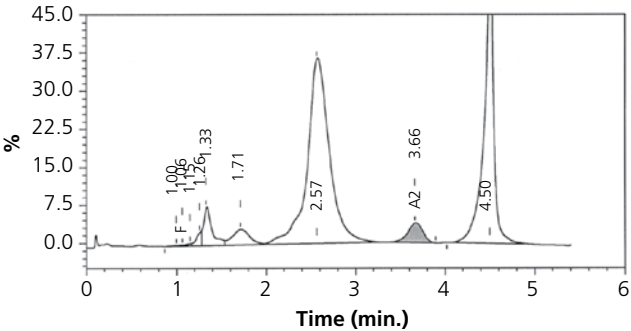
Total Area: 1,809,136

F Concentration = 0.1* %

A2 Concentration = 3.8* %

*Values outside of expected ranges

Analysis comments:



(d)

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	14.0*	---	1.17	199717
Unknown	---	1.2	2.15	17435
Ao	---	2.0	2.34	30076
A2	4.1*	---	3.66	60141
S-window	---	79.6	4.47	1200673

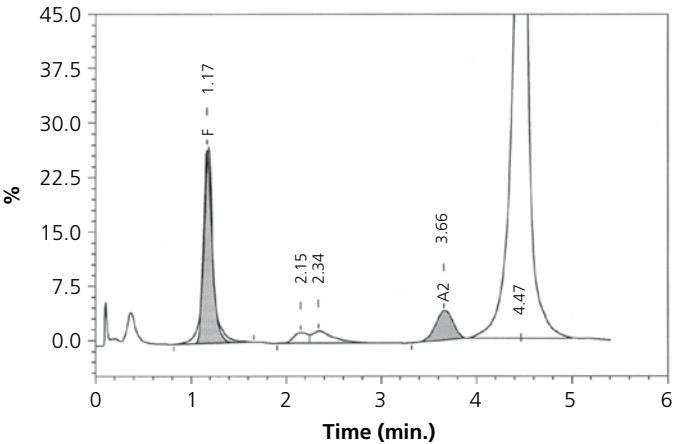
Total Area: 1,508,043

F Concentration = 14.0* %

A2 Concentration = 4.1* %

*Values outside of expected ranges

Analysis comments:



(e)

Fig. 2.19 Continued.

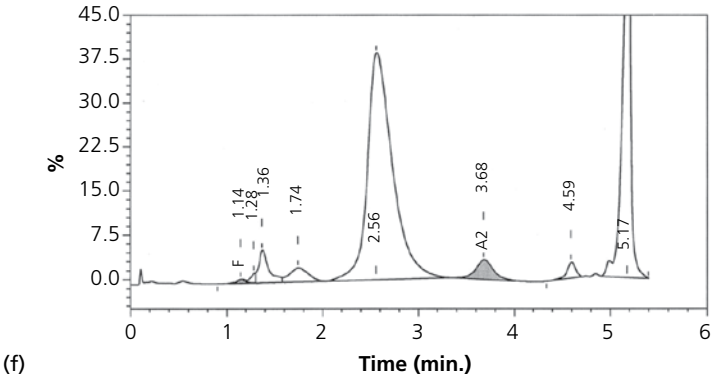
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.4	---	1.14	6283
Unknown	---	0.5	1.28	9203
P2	---	3.0	1.36	56770
P3	---	2.4	1.74	45131
Ao	---	53.5	2.56	997871
A2	3.3	---	3.68	59898
S-window	---	1.3	4.59	23544
C-window	---	35.7	5.17	664821

Total Area: 1,863,523

F Concentration = 0.4 %

A2 Concentration = 3.3 %

Analysis comments:



(f)

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.0	---	1.13	14847
P2	---	0.1	1.38	1152
P3	---	0.1	1.73	1939
Ao	---	0.9	2.58	14061
A2	4.2*	---	3.64	67438
S-window	---	2.8	4.55	46229
C-window	---	91.2	5.14	1504060

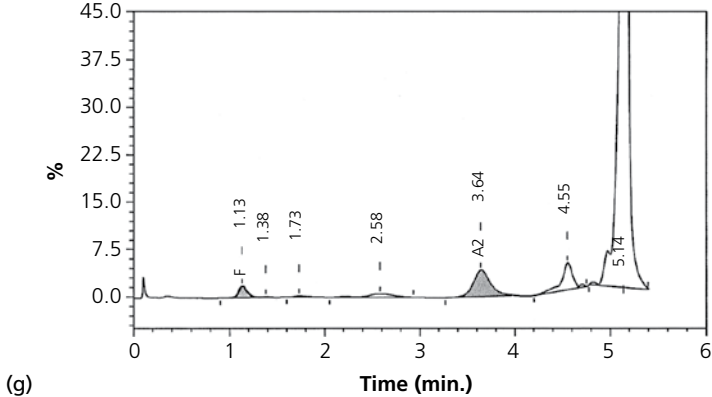
Total Area: 1,649,725

F Concentration = 1.0 %

A2 Concentration = 4.2* %

*Values outside of expected ranges

Analysis comments:



(g)

Fig. 2.19 Continued.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.5	2.13	8629
Ao	---	0.9	2.30	16416
A2	4.7*	---	3.64	77857
S-window	---	48.6	4.52	908723
C-window	---	45.9	5.16	856823

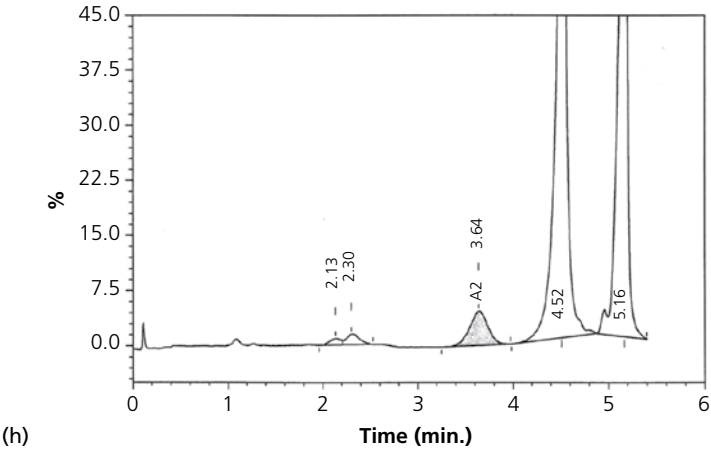
Total Area: 1,868,449

F Concentration = %

A2 Concentration = 4.7* %

*Values outside of expected ranges

Analysis comments:



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.7	---	1.09	12880
Unknown	---	1.2	1.21	24602
P2	---	5.5	1.28	111750
P3	---	6.4	1.71	129925
Ao	---	60.1	2.48	1214728
A2	25.1*	---	3.67	525652

Total Area: 2,019,538

F Concentration = 0.7 %

A2 Concentration = 25.1* %

*Values outside of expected ranges

Analysis comments:

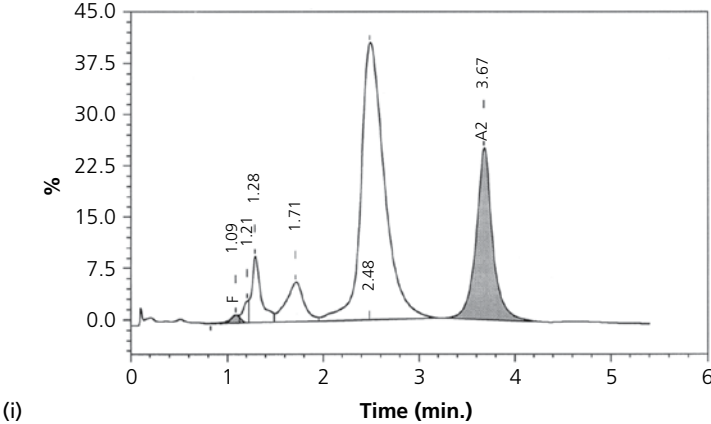


Fig. 2.19 Continued.

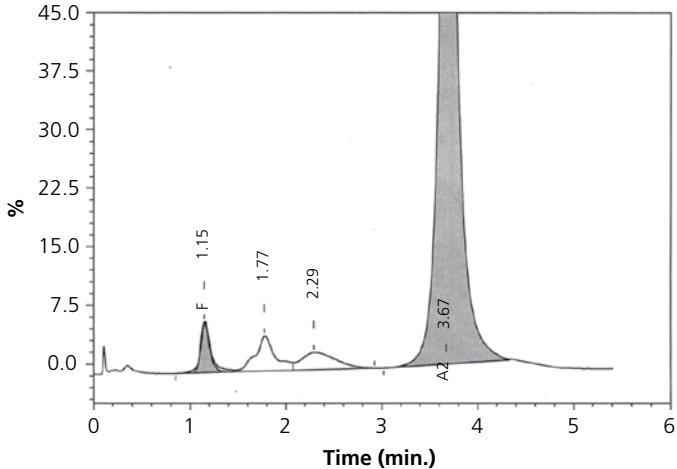
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	3.2*	---	1.15	43652
P3	---	4.7	1.77	68314
Ao	---	4.0	2.29	57579
A2	90.1*	---	3.67	1277484

Total Area: 1,447,028

F Concentration = 3.2* %
A2 Concentration = 90.1* %

*Values outside of expected ranges

Analysis comments:



(j)

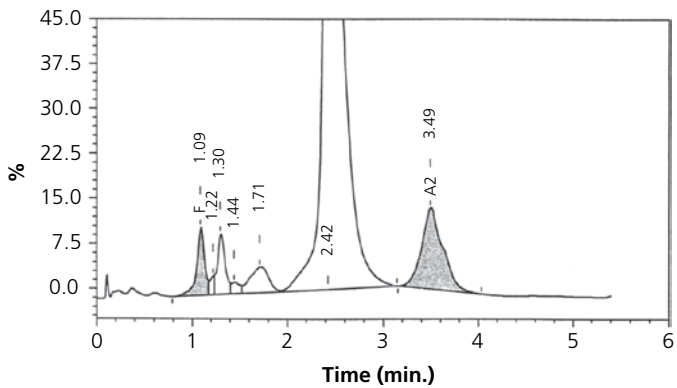
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	3.7*	---	1.09	66822
Unknown	---	0.6	1.22	11336
P2	---	3.1	1.30	56593
Unknown	---	0.7	1.44	12536
P3	---	3.2	1.71	58222
Ao	---	77.0	2.42	1390478
A2	13.4*	---	3.49	210129

Total Area: 1,806,116

F Concentration = 3.7* %
A2 Concentration = 13.4* %

*Values outside of expected ranges

Analysis comments:



(k)

Fig. 2.19 Continued.

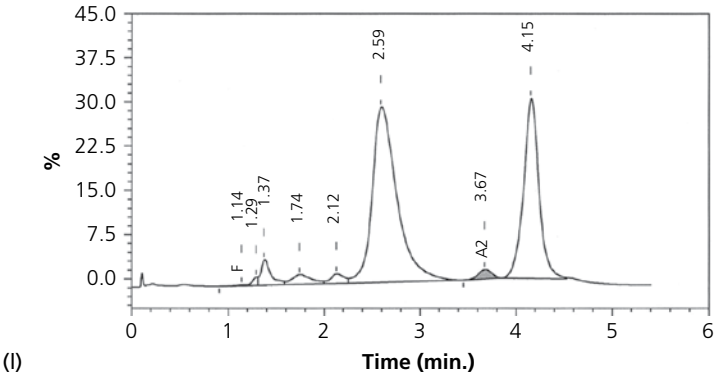
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.1*	---	1.14	1735
Unknown	---	0.6	1.29	7602
P2	---	3.2	1.37	40510
P3	---	2.4	1.74	30771
Unknown	---	1.7	2.12	20844
Ao	---	55.2	2.59	695575
A2	1.6*	---	3.67	19397
D-window	---	35.2	4.15	444023

Total Area: 1,260,457

F Concentration = 0.1* %
A2 Concentration = 1.6* %

*Values outside of expected ranges

Analysis comments:



(l)

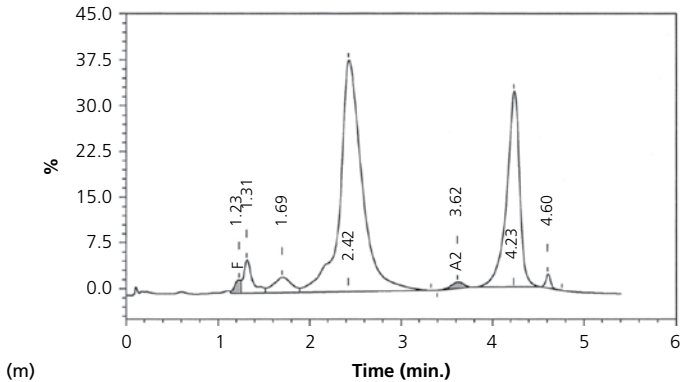
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.0	---	1.23	25854
P2	---	3.4	1.31	88450
P3	---	3.2	1.69	83353
Ao	---	60.6	2.42	1560836
A2	1.0*	---	3.62	23802
D-window	---	30.0	4.23	773315
S-window	---	0.8	4.60	20111

Total Area: 2,575,721

F Concentration = 1.0 %
A2 Concentration = 1.0* %

*Values outside of expected ranges

Analysis comments:



(m)

Fig. 2.19 Continued.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.0	0.59	1006
F	0.6	---	1.10	13053
Unknown	---	0.4	1.22	9331
P2	---	2.1	1.31	53260
P3	---	1.8	1.67	44229
Ao	---	41.7	2.50	1050204
A2	1.9*	---	3.65	43304
D-window	---	19.5	4.24	490450
S-window	---	1.8	4.61	46443
C-window	---	19.5	5.14	490325
Unknown	---	11.0	5.30	276746

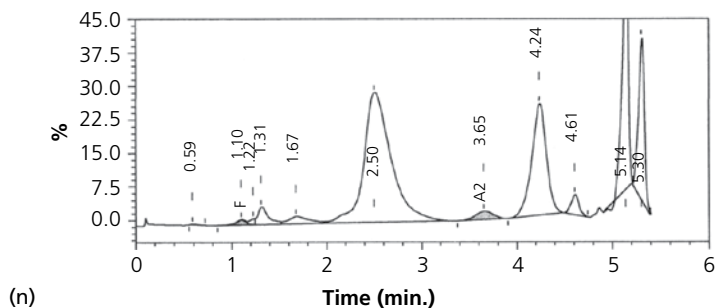
Total Area: 2,518,351

F Concentration = 0.6 %

A2 Concentration = 1.9* %

*Values outside of expected ranges

Analysis comments:



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.7	---	1.10	15518
P2	---	2.5	1.29	59235
P3	---	1.7	1.66	40329
Ao	---	51.9	2.45	1216859
A2	1.9*	---	3.63	41089
D-window	---	0.7	3.98	16074
S-window	---	0.3	4.66	8121
Unknown	---	40.4	4.89	948731

Total Area: 2,345,956

F Concentration = 0.7 %

A2 Concentration = 1.9* %

*Values outside of expected ranges

Analysis comments:

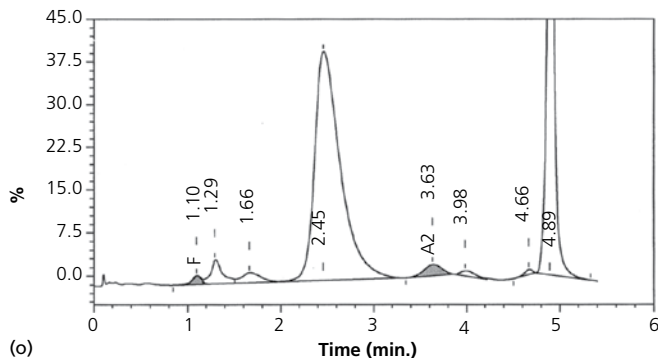


Fig. 2.19 *Continued.*

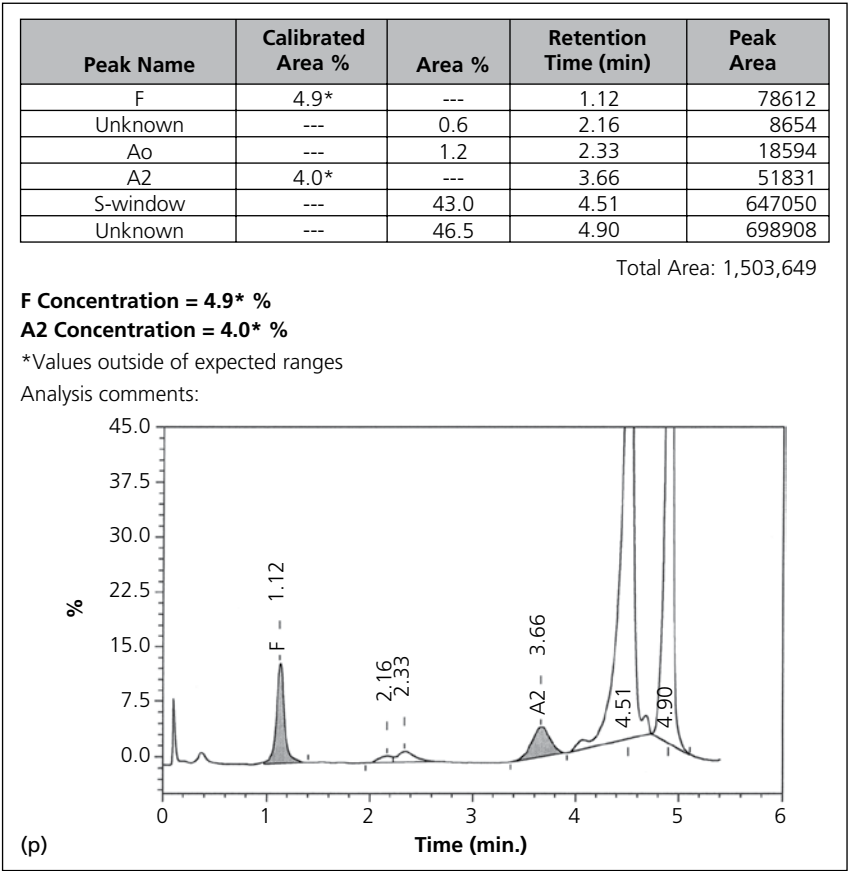


Fig. 2.19 *Continued.*

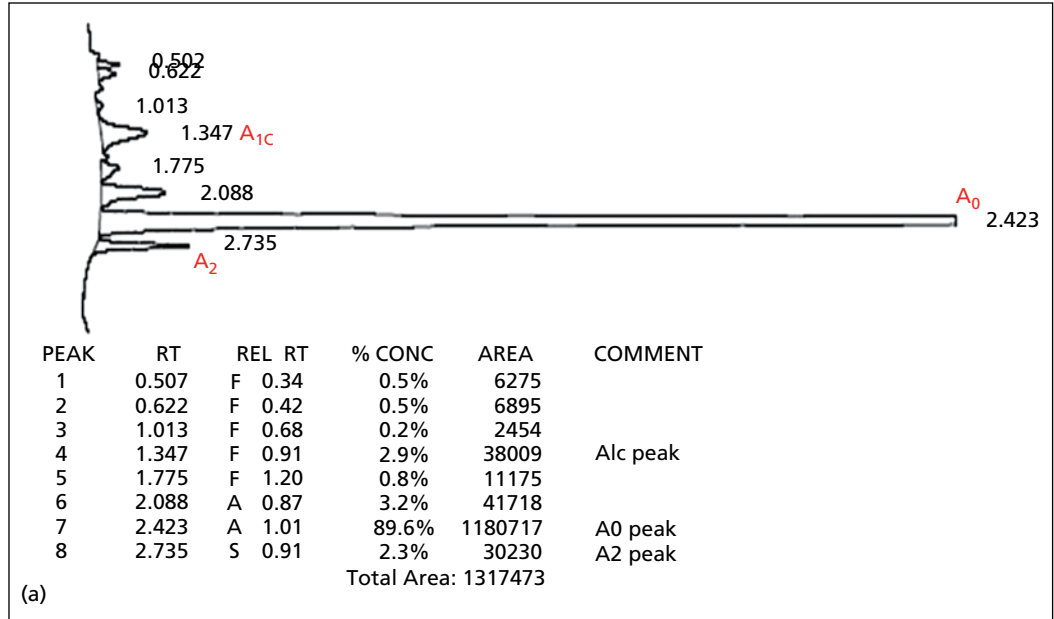


Fig. 2.20 Typical elution patterns for normal and variant haemoglobins with the Primus Ultra Plus HPLC system showing, from above down: a normal pattern (with haemoglobins A and A₂); a control sample containing haemoglobins F, A, S and C; a patient sample containing haemoglobins F, A_v, A₂ and S; a sample from a neonate with compound heterozygosity for haemoglobins S and C showing acetylated F, F_v, S and C. (With thanks to Lisa Farrell.) (*Continued on pp. 61–62.*)

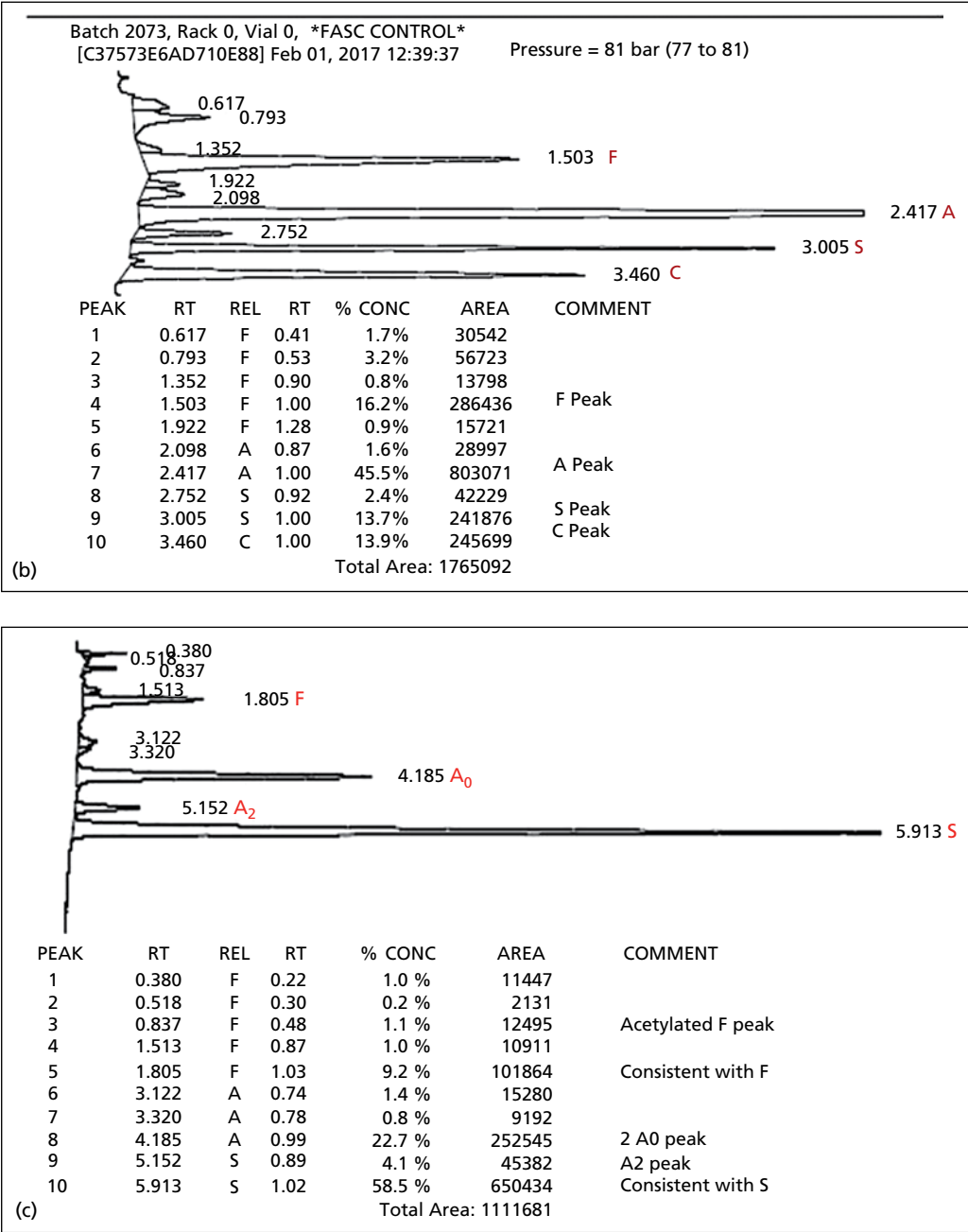


Fig. 2.20 *Continued.*

not suitable for quantification of haemoglobin A₂. IEF is a more expensive procedure than electrophoresis on cellulose acetate, both because of greater capital costs and because the cost per test is greater. It has a role in diagnosis in neonates when the ability to use a small sample volume or an eluate from a dried blood spot, together with the ability to get good separation of bands, is particularly important. In dealing with samples from adults, the advantages of isoelectric focusing over cellulose acetate electrophoresis are less important, although the ability to characterise haemoglobins

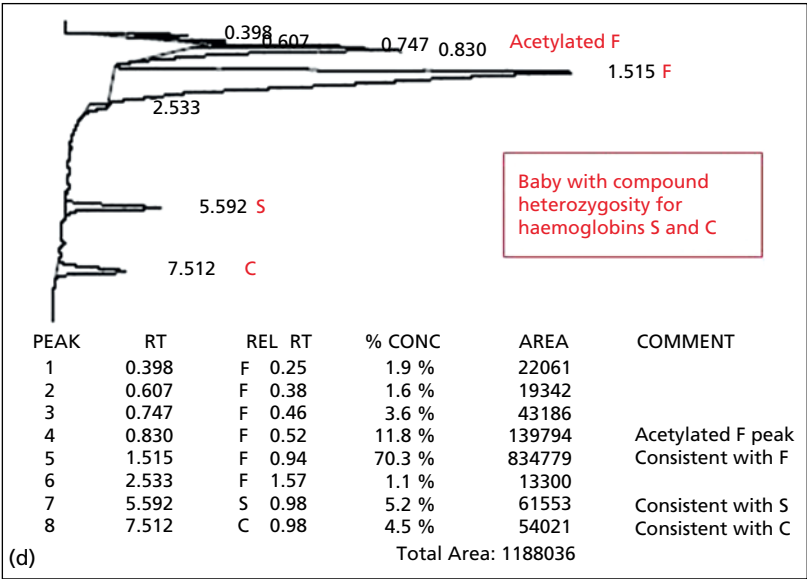


Fig. 2.20 Continued.

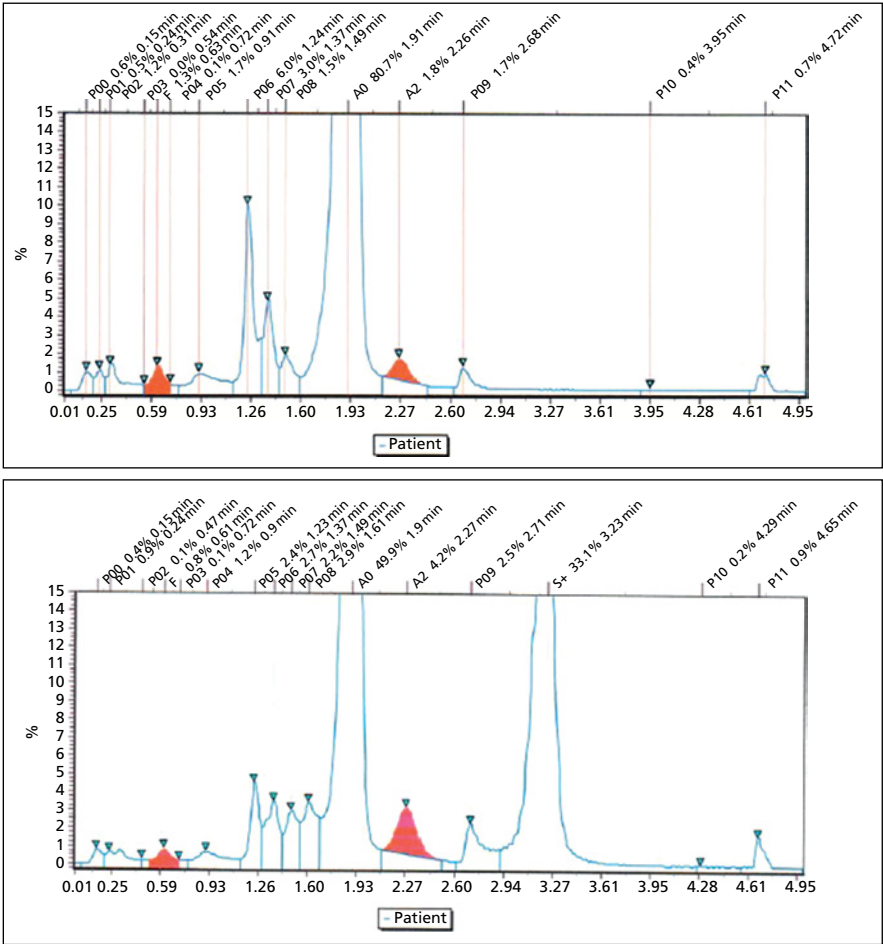


Fig. 2.21 Typical elution patterns for normal and variant haemoglobins with the Tosoh G11 HPLC system showing, from above down: a normal pattern (with haemoglobins A and A₂); sickle cell trait; haemoglobin C trait; haemoglobin D-Punjab trait; haemoglobin E trait (note that with this instrument there is separation of haemoglobins E and A₂); β thalassaemia trait (haemoglobin A₂ 4.9%). Haemoglobins F and A₂ are shown in red. (With thanks to Dr Sukhjinder Marwah.)

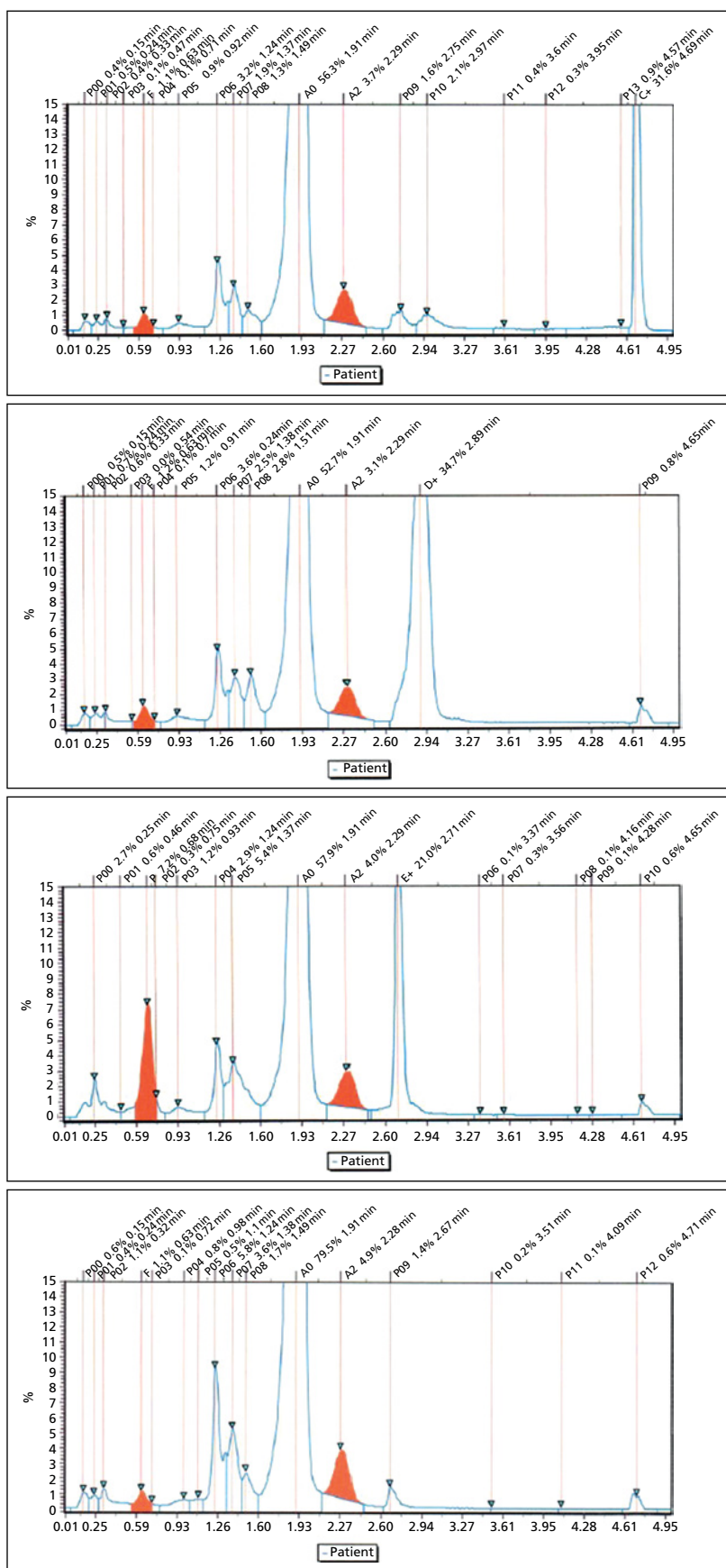


Fig. 2.21 Continued.

Table 2.3 Retention times of common normal and variant haemoglobins on the Bio-Rad Variant II system compared with other haemoglobins that may have overlapping retention times; common and diagnostically important haemoglobins are in bold. Information is from reference [33] unless otherwise cited. Some of the haemoglobins that can appear in one of two windows are listed twice.

'Window'	Retention time	Window	Haemoglobins that may overlap
F	1.10	0.98–1.22	A fraction of glycosylated haemoglobin A , J-Iran, Marseille, South Florida, Okayoma [30], Dagestan [30]
Between F and P2		1.23–1.27	A fraction of glycosylated haemoglobin A , Camperdown [30]
'P2'*	0.11	1.28–1.50	Beckman, Sherwood Forest, Raleigh, glycosylated A , K-Woolwich, Hope, I, Helsinki, and probably Osu-Christiansborg [34], Pyrgos [30], J-Abidjian [30], I-Texas [30], Olomouc [30], N-Baltimore [30], Camden [30], J-Oxford [30]
'P3'†	1.70	1.50–1.90	N-Baltimore, Camden, J-Oxford [35], Grady [30], J-Guantanamo, Andrew-Minneapolis, Hallamshire, Hopkins-II, Dublin, J-Norfolk, Le Lamentin, Zambia, Harlow, J-Rajapen, J-Habana, Santa Clara, Buffalo, Austin [35], Luton, J-Broussais, I-High Wycombe, Tatraš, J-Paris I, Gouda, J-Baltimore, Chicago [30], Hikari [30], Fukuyama [35], Fannin-Lubbock [35], J-Anatolia [35], J-Mexico [35], J-Meerut, Old Dominion, J-Toronto
A	2.50	1.90–3.10	J-Toronto [35], Detroit, Ramban, J-Calabria, J-Bangkok, K-Ibadan, Southwark, North Shore, Milne, Hofu, Athens-GA, Niigata, Seattle, San-Diego, Olympia, Johnstown, Hinwil, Ty Gard, Tacoma, Nigeria, Broomfield, Hinchingsbrook, Hekinana, P-Nilotic, Buenos Aires, North Manchester, M-Iwate, City of Hope, Alzette [30], New York, Ranier [30], Twin Peaks [35], glycosylated S, Hammersmith, Sidcup, Köln (when not denatured), Silver-Springs, Tyne, Hounslow, Fountainebleau, Ravenscourt Park, Chelsea, Bushwick, Barika, Middlesbrough
Between A and A₂		3.11–3.29	Henri Mondor [30], G-Georgia [30]
A₂	3.60	3.30–3.90	Hackney, Abruzzo, G-Coushatta, Deer Lodge, D-Iran, M-Milwaukee [30], M-Iwate [30], St Louis [30], Lepore , Kenya, Spanish Town, Fort Worth, D-Ouled Rabah, E, Ocho Rios, Cocody [30], G-Ferrara, Kenitra [30], Osu-Cristiansborg, G-Honolulu, Paddington, G-Copenhagen, M-Saskatoon‡, Zurich, Korle Bu, Alabama
D	4.10	3.90–4.30	Korle Bu [35], Alabama, Dhofar, Khartoum, D-Punjab , Oleander [30], Okazaki, Etobicoke, Caribbean, Kempsey, G-Philadelphia , West One, Atago, Maputo [30], G-Norfolk
S	4.50	4.30–4.70	G-Norfolk [30], Stanleyville II, Russ, E-Saskatoon, G-Pest, G-Waimanolo, Shimonoseki, Savaria, S , Hornchurch, Yakima, St Luke's, Ottawa, Handsworth, Q-Thailand, St Mary's, A₂' , Montgomery [35], Tarrant [30], Kokura [30], Moabit [30], glycosylated C, G ₂ , Manitoba, Winnipeg, Titusville, Haaglanden [36], Vellore [37]
Between S and C		4.71–4.89	Manitoba [30], Winnipeg [30], Titusville [30], Arya [30], Presbyterian, Setif, Q-India, C-New Cross, Hasharon, Q-Iran, O-Padova [30], S-Antilles [30], Chad [30]
C	5.10	4.90–5.30	O-Arab, O-Indonesia, Constant Spring , Agenogi, Siriraj, C

* A glycosylated fraction of haemoglobin A.
† A minor peak representing other post-translationally modified haemoglobin A.
‡ Plus a second peak in the C window.



Fig. 2.22 Photograph of isoelectric focusing (IEF) plate showing, from left to right, (a) haemoglobins F, A and Bart's (b) haemoglobins S and C; (c) haemoglobins S and F; (d) haemoglobin S; (e) haemoglobins A and D; (f) haemoglobins A and S; (g) haemoglobins A and E; (h) normal (haemoglobins A and A₂). (With thanks to Dr Barbara Wild.)

D and G more precisely can be useful. Isoelectric focusing has the disadvantage that minor components such as methaemoglobin (resulting from ageing of the sample) and glycosylated haemoglobins are resolved as separate bands and this adds to the complexity and difficulty in interpretation. For routine use in adults, HPLC is a much more useful technique.

Capillary isoelectric focusing

In this technique IEF is carried out in a capillary tube, permitting higher voltages to be used and shortening the run time. Haemoglobins are focused at their pI and are then mobilised to pass through a detector. Capillary IEF can be automated.

Sickle solubility test and other methods for the detection of haemoglobin S

Sickle test

The presence of haemoglobin S can be demonstrated by a sickle test in which sickle cell formation is induced when blood is deoxygenated.

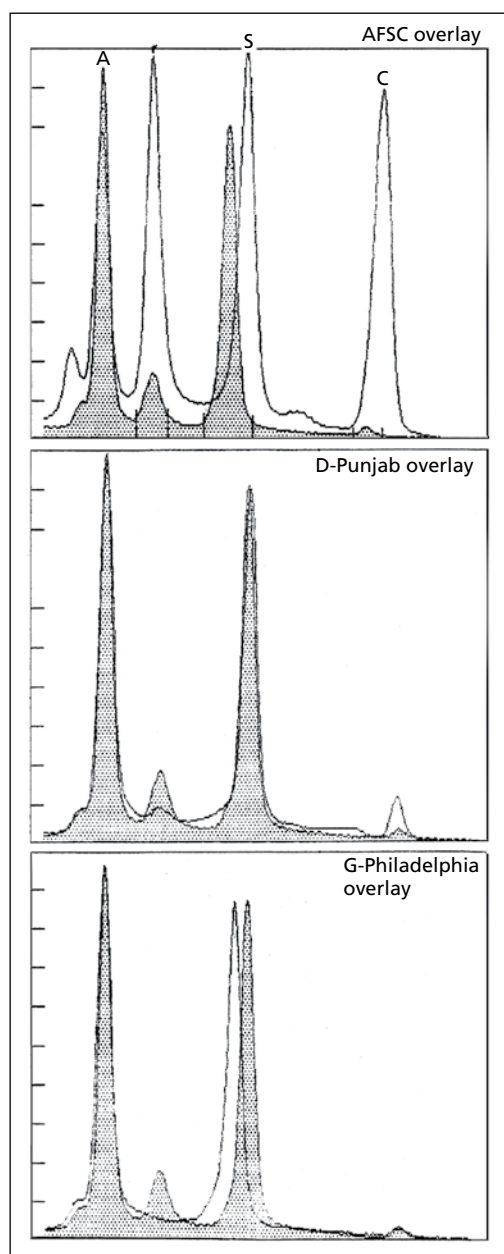


Fig. 2.23 Densitometric scanning of one lane of an IEF plate from a sample showing bands with the mobilities of A and S on cellulose acetate electrophoresis at alkaline pH. The patient's IEF scan is stippled; comparison with an A, F, S, C control sample shows that the variant haemoglobin is not S; comparison with control results for haemoglobin D-Punjab and haemoglobin G-Philadelphia identifies the variant as haemoglobin D-Punjab.

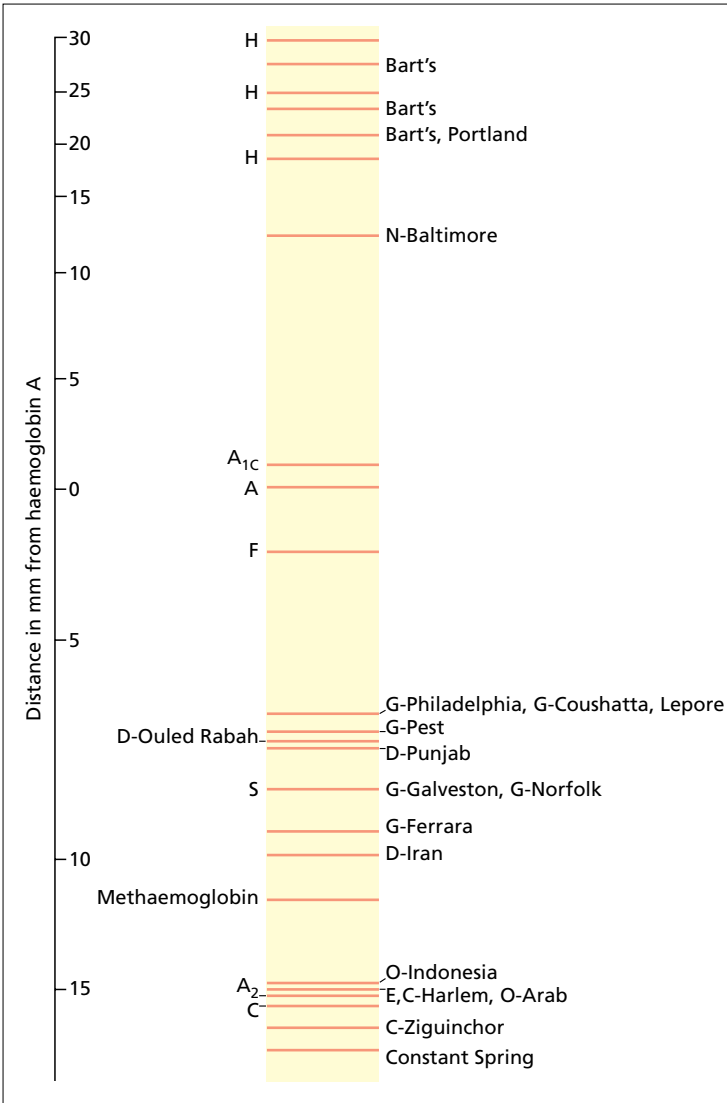


Fig. 2.24 Diagram showing the mobilities of various haemoglobins on an IEF plate: haemoglobins with similar mobility on haemoglobin electrophoresis can be distinguished from each other: haemoglobins A₂, C and E can be distinguished (but E cannot be distinguished from C-Harlem and O-Arab): haemoglobins S, D-Punjab and G-Philadelphia can be distinguished from each other (and also from D-Iran and G-Galveston but G-Philadelphia has the same pI as G-Coushatta and Lepore). (Modified from reference [35].)

A drop of blood is sealed between a glass slide and a cover slip and is sealed with molten paraffin wax so that the metabolic activity of white cells leads to deoxygenation. After an appropriate period of time, the preparation is observed with a microscope. In a common modification of the method, the drop of blood is first mixed with a drop of 2% sodium metabisulphite and the preparation is observed at 24 hours. These methods can be useful for teaching and research but are not very suitable for use in a routine diagnostic laboratory where their place has been taken

by a sickle solubility test. Haemoglobin C crystals may also be detected by this procedure [39].

Sickle solubility test

A sickle solubility test should be performed whenever a variant haemoglobin with the electrophoretic or HPLC characteristics of haemoglobin S is detected. The only exception is when the quantity of the variant haemoglobin present is so low that a positive sickle solubility test would be unlikely; in this circumstance an alternative

second technique should be employed to strengthen the presumptive identification of the variant haemoglobin. It is also prudent to perform a sickle solubility test whenever a variant haemoglobin of uncertain nature is present since there are a number of abnormal haemoglobins that have a second amino acid substitution in addition to the valine that replaces glutamic acid in haemoglobin S (see Table 4.2). These variant haemoglobins may have an electrophoretic mobility that differs from that of haemoglobin S but nevertheless their presence leads to red cell sickling *in vitro* and *in vivo*.

A sickle solubility test can be performed by purchasing the necessary reagents or commercial kits that include the necessary reagents [3, 40]. A number of kits have been evaluated and found to detect haemoglobin S down to a concentration of 20%, and sometimes below – in some cases as low as 8% [40]. A positive and a negative control should be included whenever a patient sample is tested (Fig. 2.25). If the patient is anaemic it is essential to correct the haematocrit to about 0.50 in order to avoid false negative tests. This can be done by removing some of the plasma but it is preferable for a sickle solubility test to be performed on reconstituted packed red cells in order to not only avoid problems from anaemia but also lessen the possibility of false positive tests when an abnormal plasma protein is present (Fig. 2.26) or when there is hyperlipidaemia. Other causes of false positive tests include a very high count of either white

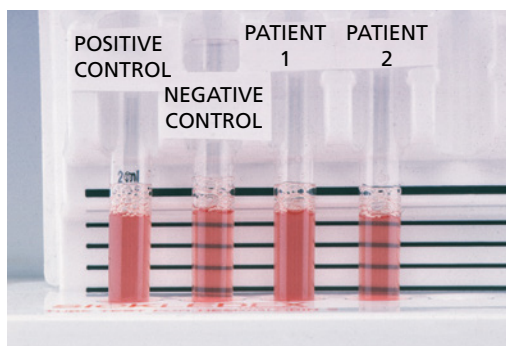


Fig. 2.25 Sickle solubility test showing a positive control, a negative control, a positive test and a negative test.

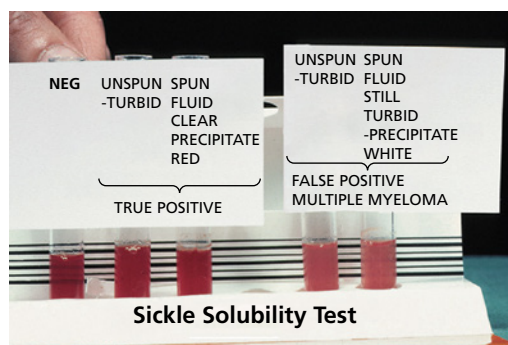


Fig. 2.26 False positive sickle solubility test caused by increased plasma proteins. From left to right, a negative control, a positive control (before and after centrifugation) and a false positive result caused by a myeloma protein (before and after centrifugation).

cells or nucleated red cells or a Heinz body haemolytic anaemia (see later). Most methods require that all negative or equivocal sickle solubility tests be centrifuged before reading to increase sensitivity and reliability. It is important that this step is not omitted if samples with a relatively low percentage of haemoglobin S are to be detected reliably. It should be noted that not only the presence of a paraprotein but also the presence of large numbers of Heinz bodies can cause a false positive sickle solubility test. The latter phenomenon can lead to a false positive test in a patient with an unstable haemoglobin, particularly if the patient has been splenectomised.

Sickle solubility tests should be capable of giving positive results in all cases of sickle cell trait beyond the period of early infancy, even when there is coexisting α thalassaemia trait. However, they should not be relied on in early infancy when the percentage of haemoglobin S may be a great deal less than 20%. In this circumstance the provisional identification of haemoglobin S should be based on two independent methods other than a sickle solubility test (e.g. IEF plus HPLC, or cellulose acetate electrophoresis plus an immunoassay).

All sickle solubility tests, whether positive, negative or equivocal, should be confirmed by haemoglobin electrophoresis or an alternative technique in order to: (i) confirm the presence of

haemoglobin S; (ii) distinguish sickle cell trait from sickle cell anaemia and from compound heterozygous states; and (iii) detect false negative results due to technical error or an unusually low percentage of haemoglobin S. If rapid results are required (e.g. before emergency anaesthesia) the distinction between sickle cell trait and sickle cell anaemia or compound heterozygous states can be made with reasonable accuracy by combining a sickle solubility test with a blood film and a blood count and assessing these in the light of clinical features (see Chapter 4).

In general, a sickle solubility test is not indicated in an infant less than six months of age since a negative result may be misleading. However, a sickle solubility test can sensibly be performed before emergency anaesthesia since, if it is negative, it is unlikely that anaesthesia will cause any clinical problems. The wording of the report on such a test must state that a negative test does not exclude the presence of a low percentage of haemoglobin S and that further testing is necessary and will follow.

Other tests for detection of sickle cell disease or haemoglobin S

A modification of the gel technology used for blood grouping has been designed for detection of haemoglobin S, the principle being that cells that have sickled do not pass through the gel; this test has been found to be unreliable and cannot be recommended [41]. Haemoglobin S can also be detected by immunoassay (see later).

Other tests have been developed that are applicable in a resource-poor setting and are discussed on page 416.

Quantification of haemoglobin A₂

Choice of method

Haemoglobin A₂ can be quantified with acceptable accuracy and precision by:

- high performance liquid chromatography;
- capillary electrophoresis;
- microcolumn chromatography;
- cellulose acetate electrophoresis followed by elution and spectrophotometry.

Elution followed by spectrophotometry is only satisfactory when relatively small numbers of samples are dealt with and its use is confined to resource poor settings. Cellulose acetate electrophoresis followed by scanning densitometry is not sufficiently precise to be recommended [42, 43]. This technique has a coefficient of variation (CV) of around 20% in comparison with a CV of 3–4% for the recommended techniques [42]. Similarly, IEF is unsuitable since it has a CV of 20% or more [44]. However, capillary IEF is satisfactory. International Council for Standardization in Haematology (ICSH) guidelines are available [45].

For the purpose of β thalassaemia diagnosis, any variant of haemoglobin A₂ (e.g. haemoglobin A₂') should be included in the quantification. When HPLC is the method used, it should be noted that haemoglobin A₂' cannot be quantified in the presence of haemoglobin S (including traces of haemoglobin S resulting from carry-over from a previous sample), haemoglobin C (since glycosylated haemoglobin C co-elutes with A₂') or haemoglobin G-Philadelphia (since haemoglobin G₂ co-elutes with A₂'). The third possibility does not create a problem since the two should be summed. A split A₂ band due to haemoglobin A₂' is otherwise detectable by HPLC (see Fig. 1.12) and is also detectable by capillary electrophoresis (Fig. 2.27).

Haemoglobin A₂ must not be confused with other haemoglobins appearing in the A₂ window on HPLC. This is relatively easy when the total of A₂ and the variant is over 10%, for example with haemoglobin E, but sometimes a variant haemoglobin is present at a very low concentration, e.g. haemoglobin G-Fort Worth, and this can lead to misinterpretation. Occasionally, cases of thalassaemia intermedia are associated with high levels of haemoglobin A₂ greater than 10%, and accurate diagnosis may involve family studies or deoxyribonucleic acid (DNA) analysis. Haemoglobin A₂ will be falsely low if an A₂ variant falls in the A₀ window; this is the case with haemoglobin J-Meerut, which can cause diagnostic problems when there is coinheritance of β thalassaemia trait.

The standardisation of quantification of haemoglobin A₂ by HPLC and capillary electrophoresis

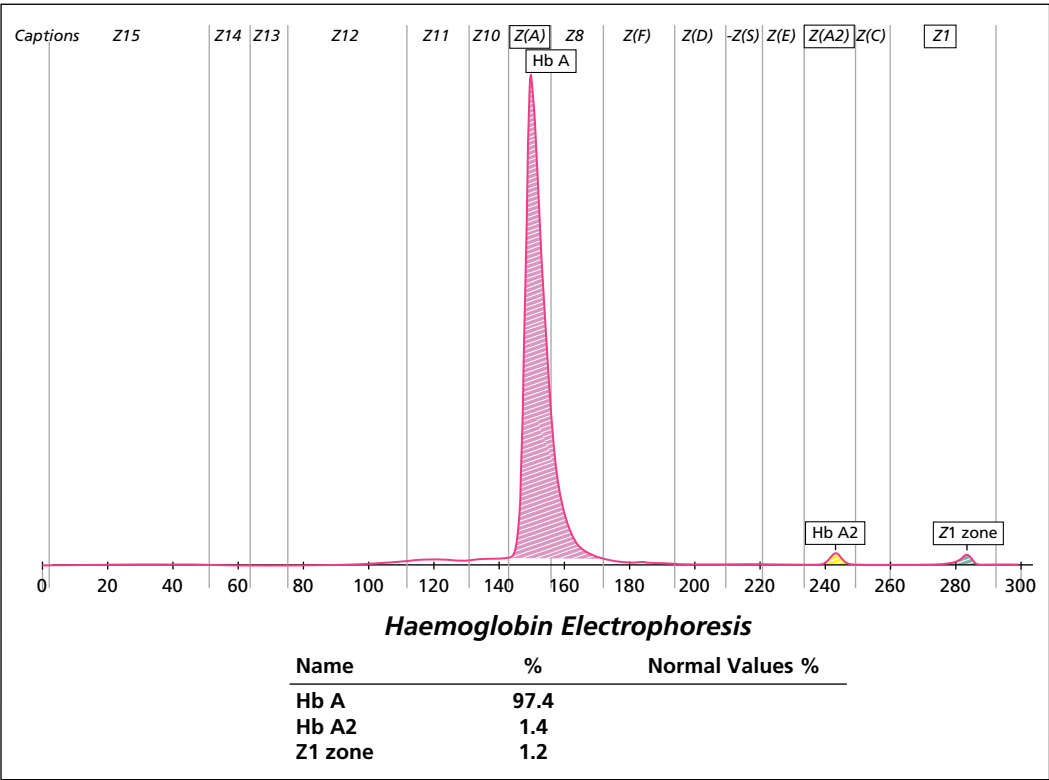


Fig. 2.27 Capillary electrophoresis, electropherogram of a sample from a patient with a delta chain mutation, Sebia Capillarys 3, showing haemoglobins A, A₂ and A₂'. Note that the sum of A₂ and A₂' is normal.

remains imperfect. There is both variation in mean values on the same samples between different instruments, sometimes even different instrument from the same manufacturer [28, 46, 47], and also variation in the degree of imprecision. In one study of 40 samples, mean values varied from 2.7% to 3.1% between five HPLC instruments and between 2.4% and 3.0% for three capillary electrophoresis instruments [48]. The CV of duplicate measurements varied from 0.5% to 4.4% [48]. In a comparison of nine instruments (either HPLC or capillary electrophoresis) some instruments produced high outliers and others low outliers [49].

High performance liquid chromatography

This is now the method most often used for the quantification of haemoglobin A₂. In general, it is a satisfactory method but haemoglobin A₂

may be underestimated in the presence of haemoglobin D-Punjab [35, 50] (as a result of partial overlap and a high baseline) (Fig. 2.28a,b) and overestimated in the presence of haemoglobin S [51] (because of the presence of post-translationally modified haemoglobin S in the A₂ window). Similarly, haemoglobin A₂ appears as a double peak in the presence of haemoglobin Q-India, attributable to glycated Q-India and likely to give rise to some overestimation; this is counterbalanced by the presence of a variant A₂ with a Q-India α chain (Fig. 2.29). With some instruments (e.g. Bio-Rad Variant II) haemoglobin A₂ cannot be measured in the presence of haemoglobin E while with others (e.g. Primus Ultra II and Tosoh G11) coelution does not occur. Overestimation of haemoglobin A₂ in the presence of haemoglobin C has been reported with some instruments (Menarini, Tosoh and Beckman) [48].

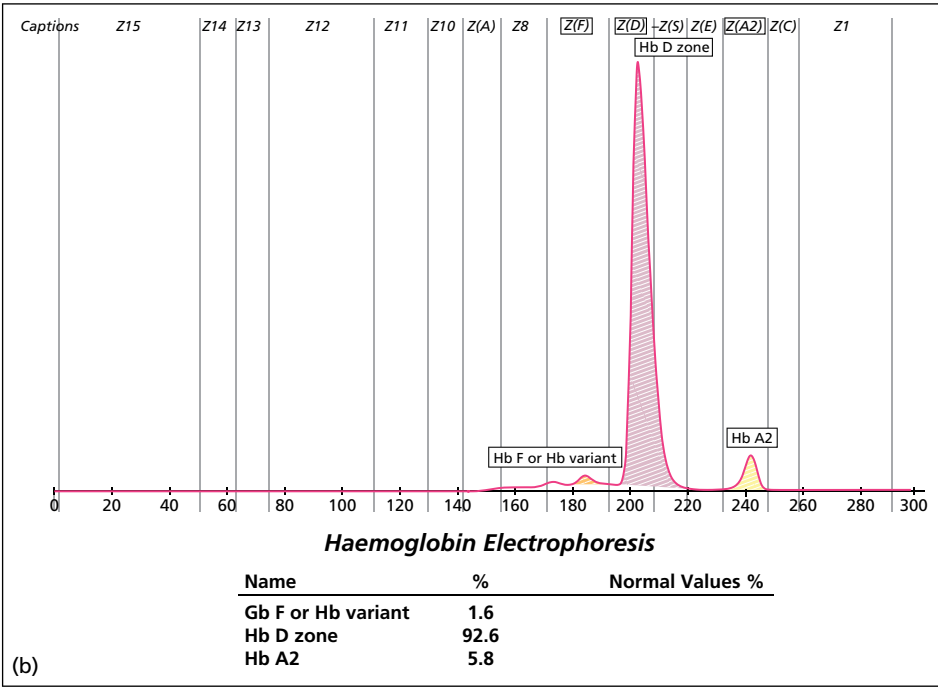
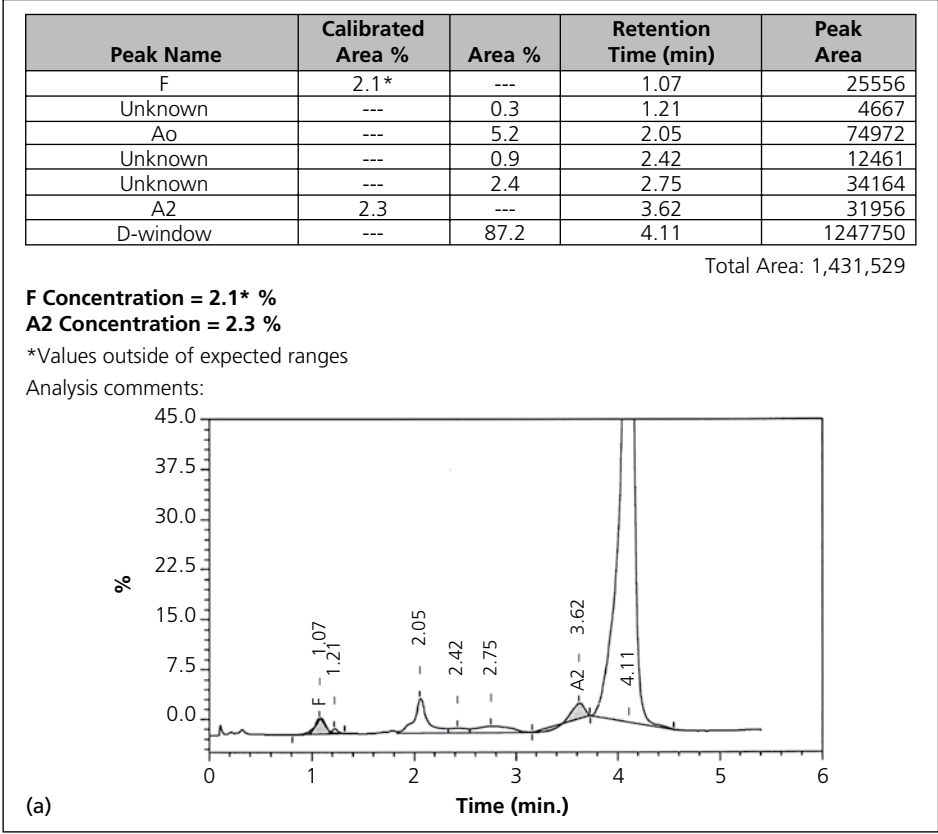


Fig. 2.28 Traces from a patient with haemoglobin D-Punjab/ β^0 thalassaemia: (a) HPLC chromatogram (Bio-Rad Variant II) showing that haemoglobin A₂ has been seriously underestimated at 2.3%; (b) capillary electrophoresis showing that haemoglobin A₂ has been correctly estimated at 5.8%.

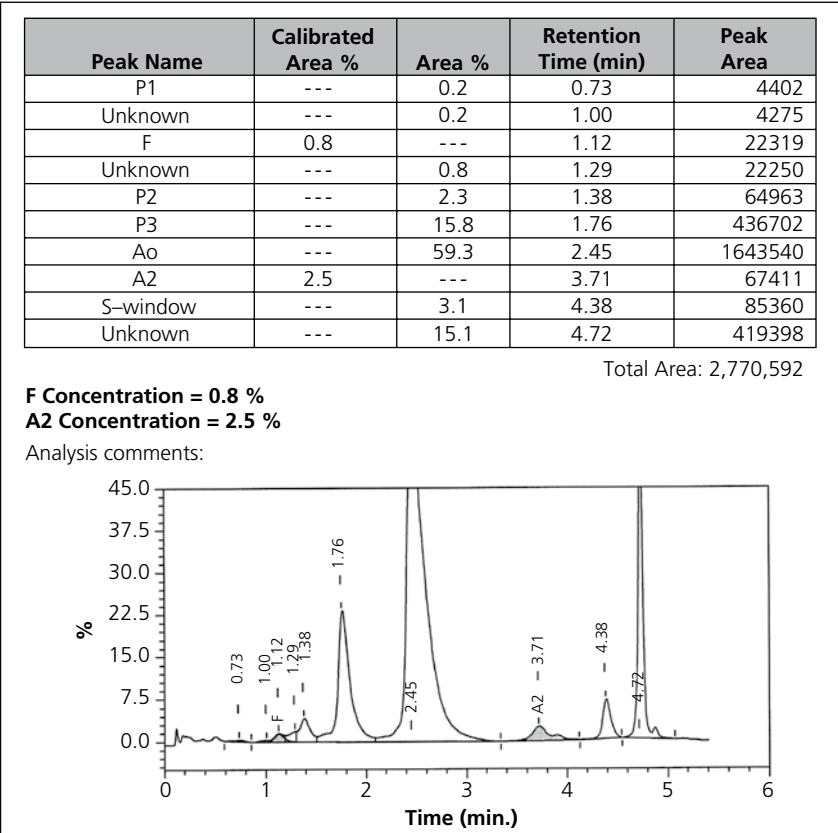


Fig. 2.29 HPLC chromatogram (Bio-Rad Variant II) from a carrier of haemoglobin Q-India. The peaks from left to right are: injection artefact, haemoglobin F, glycated haemoglobin A, other post-translationally modified haemoglobin A, haemoglobin A₀, double peak representing haemoglobin A₂ plus glycated Q-India, other post-translationally modified haemoglobin Q-India, unmodified haemoglobin Q-India and a variant haemoglobin A₂ with a Q-India α chain.

Capillary electrophoresis

Capillary electrophoresis can be used to quantify haemoglobin A₂. The method shows satisfactory precision and good correlation with HPLC, although mean values show slight but statistically significant differences. In one study the mean value for haemoglobin A₂ with Sebia Capillars electrophoresis was higher than that with HPLC using a Primus instrument (mean values 2.8% and 2.3% respectively) [9]. Similarly, in a comparison with a Bio-Rad Variant II instrument, mean values in normal subjects were 2.95% by HPLC in comparison with 2.49 with a Sebia Capillars II instrument [50];

in a second study with the same instruments, mean values were 3.27 by HPLC and 3.17 by capillary electrophoresis ($p < 0.03$) [10].

Capillary electrophoresis is more satisfactory than microcolumn chromatography in patients with sickle cell trait [50, 52] or haemoglobin D trait [50]. In the presence of haemoglobin S, the measured haemoglobin A₂ is lower by capillary electrophoresis than by HPLC (mean values 3.1% and 4% respectively) [9]. The reverse is seen in the case of haemoglobin D, in which haemoglobin A₂ is underestimated on HPLC. Measurement is possible in the presence of haemoglobin E but not always in the presence of haemoglobin C [10].

Microcolumn chromatography

Microcolumn chromatography is an anion-exchange chromatography method, which has now largely been replaced by other methods. Microcolumns are prepared containing a suspension of an anion-exchange resin in buffer. The resin is composed of small particles of cellulose covalently bound to small positively charged molecules. A haemoglobin solution is applied to the column and is adsorbed onto the resin. There is then an interchange of charged groups between the positively charged resin and the negatively charged haemoglobin molecules, which retards the passage of haemoglobin through the column. The strength of the association of various types of haemoglobin molecule to the matrix can be altered by changing the pH and ionic strength of the eluting solution applied to the column. It is therefore possible to elute different haemoglobins selectively by using different eluting solutions (Fig. 2.30). When this method is used for quantification of haemoglobin A₂ there is elution first

of haemoglobin A₂ and then, using a second eluting solution, of haemoglobin A. The two fractions are collected separately and the absorbance of the eluate is read on a spectrophotometer, permitting expression of the amount of haemoglobin A₂ present as a percentage of total haemoglobin. Alternatively, it is possible to elute only haemoglobin A₂ and measure total haemoglobin in a second tube.

Microcolumn chromatography is a satisfactory technique for quantification of haemoglobin A₂ unless relatively large numbers of samples are to be assayed. Chromatography columns can be prepared by individual laboratories.

It should be noted that the standard column for measurement of haemoglobin A₂ in suspected β thalassaemia trait is not suitable for haemoglobin A₂ quantification in the presence of haemoglobin S. Modified columns for this purpose can be prepared.

It should, however, be noted that measuring haemoglobin A₂ in the presence of haemoglobin S is not essential for making a distinction between sickle cell/ β^+ thalassaemia and sickle cell trait.

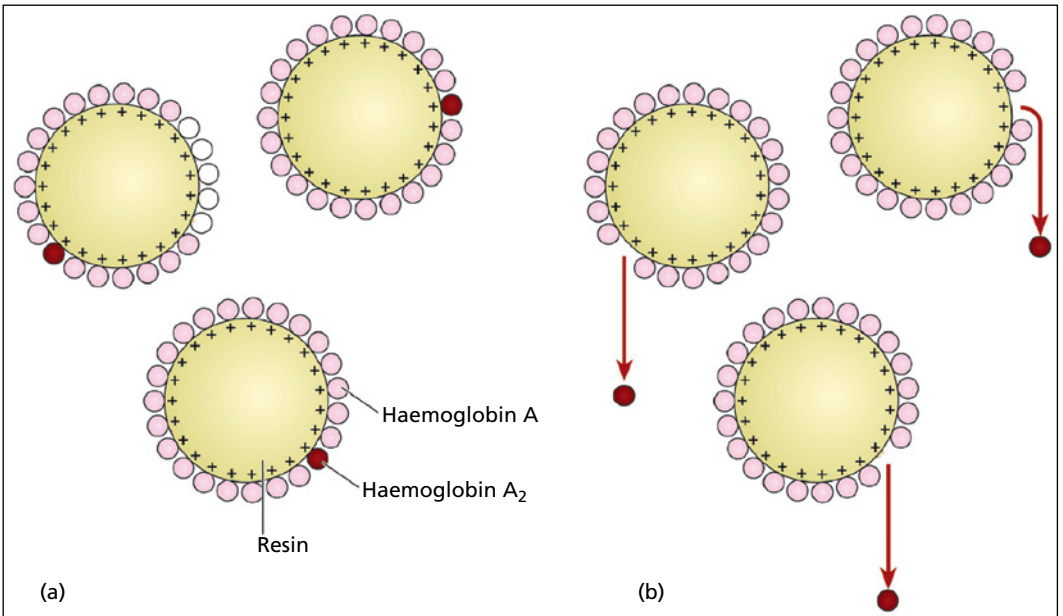


Fig. 2.30 Diagrammatic representation of the principle of microcolumn chromatography: (a) both haemoglobin A (pink) and haemoglobin A₂ (dark red) are negatively charged and are bound to the positively charged beads of the anion-exchange resin; (b) with application of an eluting solution that alters pH and ionic strength, haemoglobin A₂ is eluted while haemoglobin A remains attached to the matrix beads.

This distinction can be more readily made by quantifying haemoglobin S since S is more than 50% of total haemoglobin in sickle cell/ β^+ thalassaemia and less than 50% in sickle cell trait. Expressed in another way, there is more haemoglobin A than S in sickle cell trait and more haemoglobin S than A in sickle cell/ β^+ thalassaemia. Measuring haemoglobin A₂ in the presence of haemoglobin S is of limited value in distinguishing between sickle cell anaemia and sickle cell/ β^0 thalassaemia (see pages 243 and 262). It should be noted, in addition, that coexisting α thalassaemia trait will influence these values. Overlap occurs between these two conditions and interpretation should be undertaken with caution. The normal range of haemoglobin A₂ in the presence of haemoglobin S has not been defined but in general, quantifying haemoglobin A₂ in the presence of haemoglobin S is not a test that a laboratory needs to perform.

Microcolumn chromatography should be combined with haemoglobin electrophoresis at alkaline pH in order to detect any variant haemoglobin. It should be noted that an increased haemoglobin A₂ percentage can occur in the presence of an unstable haemoglobin and if there is any reason to suspect that a case is not a straightforward β thalassaemia trait, a specific test for an unstable haemoglobin should be performed.

A reference range should be determined in each individual laboratory, using blood samples from healthy subjects with a normal haemoglobin concentration and normal red cell indices. Results just above the upper limit of normal (e.g. 3.4–3.7%) should be regarded as equivocal, should be interpreted in the light of clinical and haematological features and should usually be repeated on the same sample and also on a second sample.

Quantification and determination of distribution of haemoglobin F

Quantification

Quantification of haemoglobin F is useful in the diagnosis of thalassaemia and hereditary persistence of fetal haemoglobin, and in

monitoring response to treatment in sickle cell anaemia. An ICSH guideline is available [53]. Unlike measurements of haemoglobin A₂, there are no precisely defined diagnostic ranges. Quantification can be by HPLC, capillary electrophoresis or capillary IEF. In a resource-poor setting, cellulose acetate electrophoresis followed by scanning densitometry or elution is applicable. Two-minute alkali denaturation followed by spectrophotometry (modified Betke method) can also give a clinically useful result. Scanning densitometry is unreliable below 10–15% and either HPLC or alkali denaturation [3] is then preferred to densitometry. HPLC gives estimates somewhat higher than alkali denaturation [29, 31]. For levels above 50%, scanning densitometry, elution plus spectrophotometry or HPLC is preferred as alkali denaturation is inaccurate. Haemoglobin F can also be quantified by an immunoassay, using radial immunodiffusion but this method has been found to be inaccurate [54] and therefore cannot be recommended. A bias between methods is expected since HPLC does not integrate post-translationally modified haemoglobin F with F whereas with methods based on electrophoresis, these fractions have the same characteristics as haemoglobin F. In one study of 82 patients with haemoglobin S and increased haemoglobin F, the mean haemoglobin F was 11.2% by capillary electrophoresis and 9.3% by HPLC, the difference being statistically significant [55].

High performance liquid chromatography

Chromatograms should be examined carefully if haemoglobin F is apparently increased on HPLC since an increased glycosylated haemoglobin is sometimes misidentified as haemoglobin F [4]. It should be noted that, with some HPLC instruments, acetylated haemoglobin F (F₁) appears in the void volume and is not integrated so that total haemoglobin F is underestimated. If haemoglobin F appears to be greater than 10% on HPLC, its nature should be confirmed by an alternative test to exclude misidentification of haemoglobin N or haemoglobin J as haemoglobin F [4].

Capillary electrophoresis

Acetylated and other adducts of haemoglobin F do not separate from F₀ so that all are included in the quantification.

Distribution of haemoglobin F

The distribution of haemoglobin F between individual red cells can be determined by the Kleihauer test or, more reliably, by flow cytometry.

Kleihauer test

A Kleihauer test is useful for confirmation whenever there appears to be an increased percentage of haemoglobin F. The distribution of haemoglobin F between cells can help to distinguish $\delta\beta$ thalassaemia trait, when the distribution of haemoglobin F is usually heterocellular, from many cases of hereditary persistence of fetal haemoglobin, in which the distribution of haemoglobin F is pancellular (see Table 3.14). The Kleihauer test is also applicable to the detection of fetomaternal haemorrhage. In this setting, it is important to distinguish deep pink fetal cells from the paler pink cells of a mother with hereditary persistence of fetal haemoglobin [56] (Fig. 2.31).

Quantification of cells containing haemoglobin F

Cells containing haemoglobin F (F cells) can be quantified by flow cytometry using permeabilised red cells and a fluorochrome-conjugated monoclonal antibody to haemoglobin F [57]. This method is applicable to the quantification of fetomaternal haemorrhage as well as to the study of patients with disorders of globin chain synthesis. By combining a labelled antibody to haemoglobin F with a fluorochrome that binds to nucleic acids, it is possible to quantitate F cells, reticulocytes and F-reticulocytes by flow cytometry [58]. This is becoming increasingly important in assessing the effects of therapies, including small molecules and gene editing, designed to increase haemoglobin F levels.

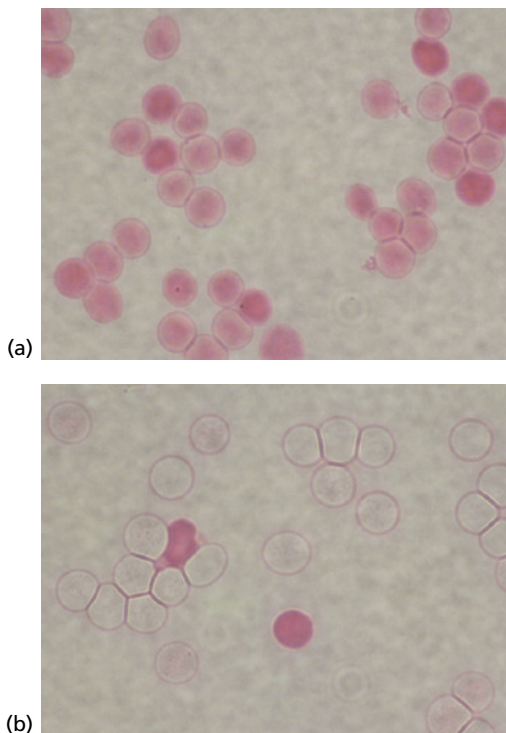


Fig. 2.31 Kleihauer test showing: (a) heterogeneous distribution of haemoglobin F in hereditary persistence of fetal haemoglobin in contrast with: (b) a control sample showing a mixture of normal and fetal cells.

Immunoassay for variant haemoglobins**Immunological detection of haemoglobin S**

Haemoglobin A and certain variant haemoglobins can be detected immunologically. Although HemoCard kits previously available for the detections of haemoglobins S, C, E and A are no longer in use, antibodies are still used in some point of care testing devices. In particular, lateral flow immunoassays have emerged using polyclonal antibodies against haemoglobins A, C and S, to allow rapid testing for sickle cell disease without further specialist equipment or trained laboratory scientists. These seem to be of particular use in low- and middle-income countries, with commercially available assays

including Sickle SCAN and HemoTypeSC being available (see Chapter 7).

Immunological detection of α^0 thalassaemia

An immunochromatographic strip has been developed in China for screening for genetic subtypes of α^0 thalassaemia in which the ζ gene is not deleted. A study in Thailand found it to be sensitive when used for screening for $-\alpha^{SEA}/\alpha\alpha$ with false positives occurring in some patients with $-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$ [59]. This technique will not detect $-\alpha^{THAI}/\alpha\alpha$ or $-\alpha^{FIL}/\alpha\alpha$ in which the ζ gene is deleted. Immunochromatographic detection of haemoglobin Bart's can also be used for screening for α^0 thalassaemia, and may not be influenced by whether or not the ζ gene is deleted [60]. An immunochromatographic strip for this purpose has also been developed in Thailand (see page 416).

Detection of haemoglobin H inclusions

A technique for the demonstration of haemoglobin H inclusions should be performed for confirmation when haemoglobin H disease is suspected and when haemoglobin electrophoresis or HPLC shows a band or peak with a mobility/retention time similar to that of haemoglobin H. Whether this test should

also be performed in suspected α^0 thalassaemia heterozygosity depends on prevalence of this condition and other resources available for testing (see page 111). It is also particularly useful in confirming cases of acquired haemoglobin H disease associated with a myelodysplastic syndrome or α thalassaemia X-linked disability syndrome, in which family studies and standard genetic diagnosis are not easily performed.

The principle of the test is that haemoglobin H precipitates following exposure to a mild oxidant such as brilliant cresyl blue. The appearance of haemoglobin H inclusions differs according to whether or not the patient has had a splenectomy. If a functional spleen is present, small blue-staining inclusions are evenly distributed through the cell, an appearance compared to a golf ball (Fig 2.32). In patients with haemoglobin H disease who have had a splenectomy, haemoglobin H is present as pre-formed Heinz bodies and, in addition, typical 'golf ball' inclusions appear during incubation with vital dyes (Fig. 2.33).

Detection of an unstable haemoglobin

Unstable haemoglobins can be detected using either a heat test or an isopropanol test. Tests should be set up with a positive and a negative control. A positive result is the appearance of a

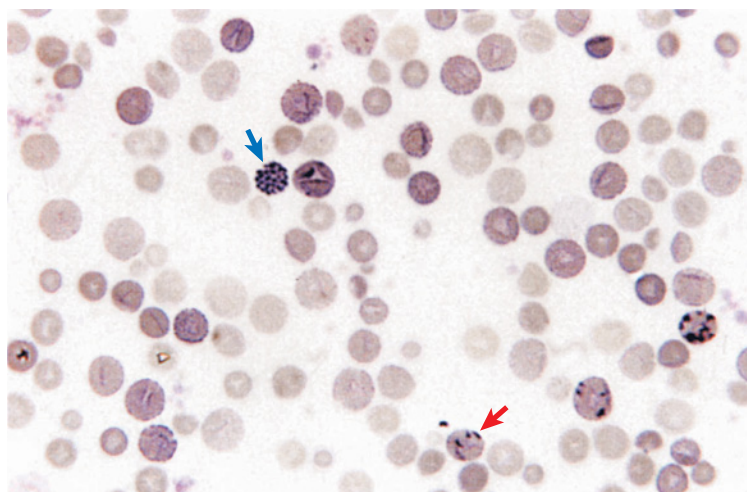


Fig. 2.32 A haemoglobin H preparation from a patient with haemoglobin H disease showing haemoglobin H inclusions (blue arrow) and reticulocytes (red arrow).

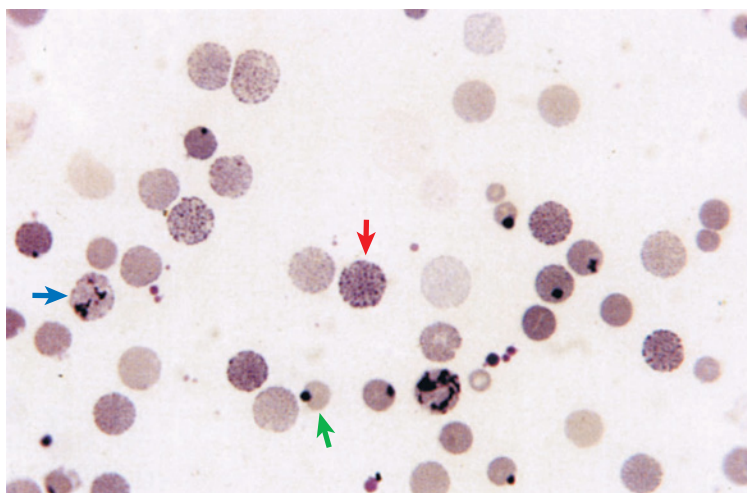


Fig. 2.33 A haemoglobin H preparation from a patient with haemoglobin H disease who had had a splenectomy showing haemoglobin H inclusions (red arrow), reticulocytes (blue arrow) and preformed Heinz bodies (green arrow).

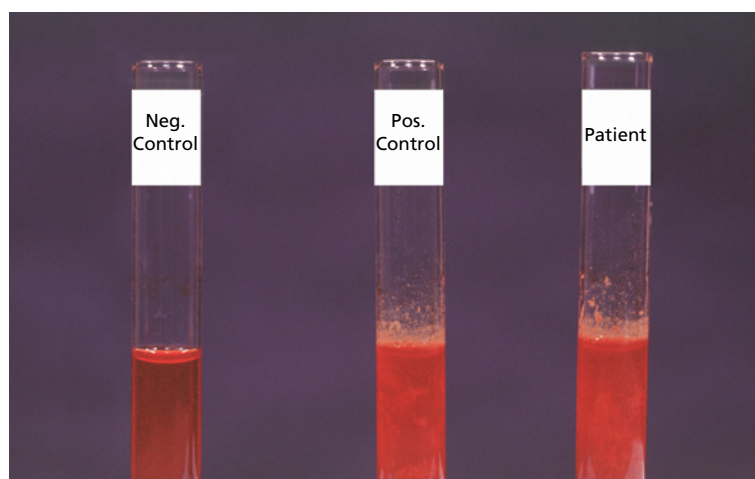


Fig. 2.34 An isopropanol test for an unstable haemoglobin.

precipitate at the end point of the test that is not present in a normal control (Fig. 2.34). The test should be performed promptly, since ageing of the blood can lead to a false positive result because of formation of methaemoglobin. If any delay has occurred, the negative control should be of the same age as the test sample. An aged fetal sample is suitable as a positive control. The fact that haemoglobin F is less stable than haemoglobin A complicates testing if an unstable fetal haemoglobin is suspected. In this circumstance a fetal sample with a similar proportion of haemoglobin F should be used as the negative control. False positive tests can result from the presence of haemoglobin S or an increased percentage of haemoglobin F.

The isopropanol test is less sensitive than the heat stability test and some slightly unstable haemoglobins do not give a positive result.

Detection of Heinz bodies

Testing for Heinz bodies, by incubation with either methyl violet or brilliant cresyl blue, is relevant whenever an unstable haemoglobin is suspected (e.g. unexplained anaemia with an increased reticulocyte count, irregularly contracted cells on a blood film or blurred bands or small irregular peaks on electrophoresis and HPLC respectively) (Fig. 2.35). If Heinz bodies are not detected in a fresh specimen, they may appear after incubation at 37°C for 24 hours. They are more likely to be present after splenectomy.

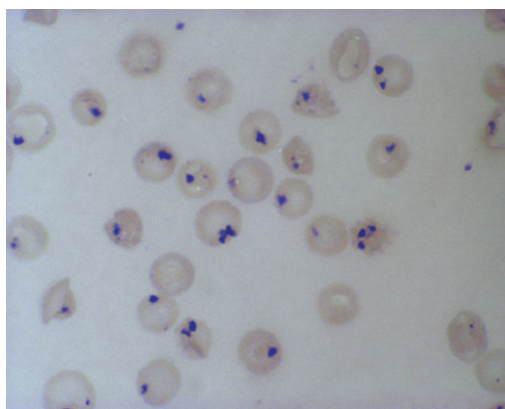


Fig. 2.35 A Heinz body preparation (incubation with methyl violet) in a patient with haemoglobin Southampton. (With thanks to Mr David Roper.)

Osmotic fragility as a screening test for thalassaemia

In resource-poor countries without easy access to automated blood counters, a simple visual one-tube osmotic fragility test has been used as a screening test for thalassaemia trait, including haemoglobin E trait [61]. This test depends on the fact that the hypochromic cells of thalassaemia trait are relatively osmotically resistant. Whereas normal cells lyse in buffered saline of a certain concentration, thalassaemia trait cells do not so that there is a cloudy suspension rather than a clear solution. Cells from individuals with iron deficiency or dehydrated hereditary stomatocytosis may also fail to lyse. The test can be done on a finger-prick sample and the number of patients requiring phlebotomy and definitive tests is thereby reduced. In one study using 0.34% saline all samples from individuals with α^0 thalassaemia were identified [62]. For β thalassaemia trait, 0.34% saline is not sufficiently sensitive, 0.36% being needed [63]. 0.45% glycerine-saline is more sensitive [64]. False positive tests (e.g. as a result of iron deficiency) are not infrequent. Coexisting South-East Asian ovalocytosis, glucose-6-phosphate dehydrogenase deficiency and α thalassaemia reduce the sensitivity of the test for the detection of β thalassaemia, possibly to as low as 70% in areas where all three disorders are common [64, 65]. The specificity of the test for β thalassaemia including haemoglobin E trait is reduced by a high frequency of iron deficiency and α thalassaemia. False positive results from iron

deficiency are a disadvantage whereas false positive results from α^0 thalassaemia are advantageous.

Dichlorophenolindophenol test for haemoglobin E screening

When resources are limited, a 2,6-dichlorophenolindophenol (DCIP) test can be used for screening for haemoglobin E, which is mildly unstable and precipitates in response to oxidative stress [66, 67] (Fig. 2.36). This reduces the number of samples that need to be referred to a central laboratory for definitive diagnosis. The test is read visually, but is harder to read than a sickle solubility test.

Quantification of haemoglobin A_{1c}

Haemoglobin A_{1c} is a glycosylated derivative of haemoglobin A. Since haemoglobin A_{1c} can be quantified by HPLC or ion-exchange microcolumn chromatography, quantification is sometimes performed by haemoglobinopathy laboratories.

The percentage of haemoglobin A_{1c} is determined by red cell life span and by the average blood glucose level during the life of the red cell. High levels are thus noted in diabetes mellitus, particularly when control is poor, and also when there has been a recent arrest of red cell production. Low levels are seen when there is a young red cell population, and this has been suggested as an aid to distinguishing haemolytic anaemia from other types of anaemia [68].

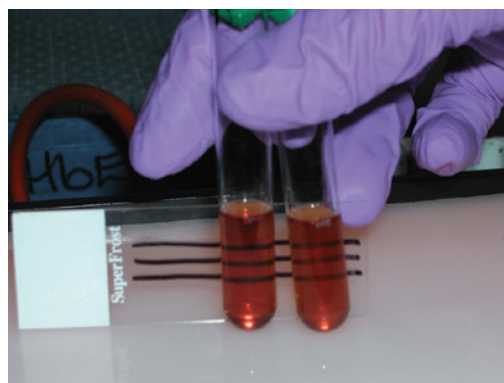


Fig. 2.36 A 2,6-dichlorophenolindophenol (DCIP) test being read on a light box with a striped glass slide to increase legibility.

Quantification has also been suggested in order to make a distinction between transient erythroblastopenia of childhood (increased percentage) and Diamond–Blackfan syndrome (normal percentage) [69]. The mean level is increased in iron deficiency, in one study the mean being 6.15% before treatment and 5.25% after treatment [70].

The percentage of haemoglobin A_{1c} , as measured by HPLC, can be erroneous in the presence of a variant haemoglobin [7, 34]. Factitious elevation can be the result of an increased percentage of haemoglobin F or the presence of a ‘fast’ haemoglobin such as haemoglobin I, J or N. Haemoglobin Hope, haemoglobin Raleigh and haemoglobin Osu-Christiansborg also have retention times similar to that of glycosylated haemoglobin A. If there is no haemoglobin A present, haemoglobin A_{1c} will necessarily be zero. Haemoglobin A_{1c} can also appear to be low if there is a variant haemoglobin present (e.g. S, C, D, G, E) and the percentage of haemoglobin A_{1c} is quantified as a percentage of total haemoglobin, ignoring the fact that there will also be a glycosylated component of the variant haemoglobin (Fig. 2.37). An alternative technique (affinity column chromatography) permits reliable quantification of haemoglobin A_{1c} despite the presence of a variant haemoglobin.

A high glycosylated fraction may be noted during investigation of a suspected variant haemoglobin. If the patient is *not* known to suffer from diabetes, the presence of an elevated level should be reported and definitive testing for diabetes is then appropriate [22].

Incidental detection of a variant haemoglobin

Incidental detection and subsequent identification of a variant haemoglobin, not necessarily of any clinical significance, is quite frequent when HPLC is used for quantification of haemoglobin A_{1c} and aberrant results are noted. Much less often, a variant haemoglobin is detected because of a factitiously low arterial oxygen saturation by pulse oximetry. The factitiousness of the result is identified when a measurement of arterial oxygen saturation is normal. The explanation is that the oxy form of the variant haemoglobin has an absorption peak close to the absorption trough of oxyhaemoglobin A at 660 nm, giving a falsely reduced difference between the measured peak and the measure trough [71]. Similarly, haemoglobin variants resulting in increased methaemoglobin levels or altered oxygen affinity may give misleading results on pulse oximetry.

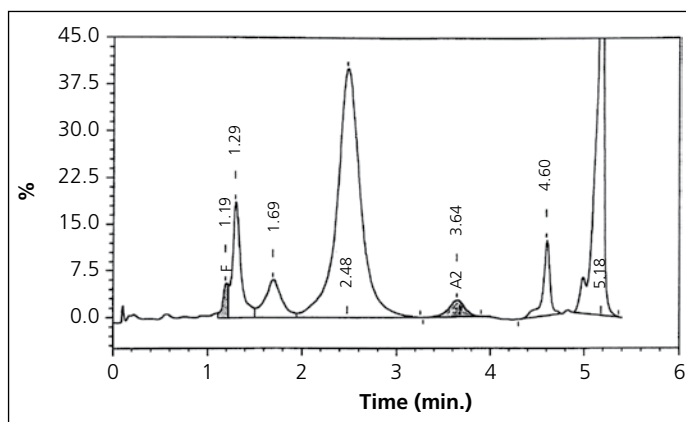


Fig. 2.37 HPLC chromatogram (Bio-Rad Variant II) from a patient with haemoglobin C trait showing glycosylated haemoglobin A and glycosylated haemoglobin C: from left to right the peaks are haemoglobin F (shaded), glycosylated haemoglobin A (retention time 1.29 minutes), other post-translationally modified haemoglobin A, haemoglobin A_{1c} , haemoglobin A_2 (shaded), glycosylated haemoglobin C (retention time 4.6 minutes) and haemoglobin C with a shoulder on the left of the peak that represents other post-translationally modified haemoglobin C.

Other more specialised tests

Some more specialised tests are required infrequently and are generally better performed in a regional centre or reference laboratory rather than in a routine diagnostic laboratory. The exception is in certain geographic areas where a high prevalence of specific disorders of haemoglobin synthesis makes it cost effective for laboratories in large hospitals to carry out specific specialised tests. For completeness, these tests will be discussed briefly in this chapter.

Detection of high or low affinity haemoglobins

An oxygen dissociation curve with determination of p_{50} (the pO_2 at which haemoglobin is 50% saturated) should be performed if a high oxygen affinity haemoglobin is suspected (Fig. 2.38). Low affinity haemoglobins (other than haemoglobin S) are quite uncommon so that although they also are diagnosed by an oxygen dissociation curve, this investigation is rarely productive in the investigation of an unexplained low haemoglobin concentration. High affinity haemoglobin variants are often electrophoretically silent, and DNA analysis, such as sequencing of the α and β globin genes, is indicated if this is suspected.

Globin chain electrophoresis

Globin chain electrophoresis (Fig. 2.39) is carried out on a red cell lysate to which DL-dithiothreitol and urea have been added to dissociate the haem groups and globin chains. Electrophoresis is then carried out on cellulose acetate membranes using both acid and alkaline buffer systems. Globin chain electrophoresis permits a distinction between α and β chain abnormalities and, when used as a supplement to haemoglobin electrophoresis, permits a presumptive identification of a larger range of variant haemoglobins. This technique has become unimportant since the wider availability of HPLC has provided an alternative technique that is much more rapid and less labour intensive.

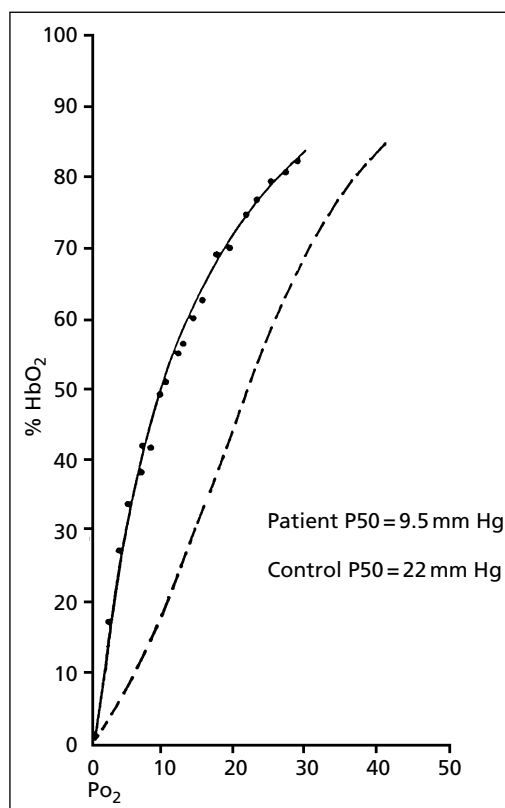


Fig. 2.38 An oxygen dissociation curve showing haemoglobin A (broken line) and a high affinity haemoglobin, haemoglobin Heathrow (complete line). (With thanks to Mr David Roper.)

Analysis of the rate of globin chain synthesis

The relative rates of synthesis of α and β globin chains by reticulocytes or bone marrow cells can be useful in the diagnosis of thalassaemias. This is determined by the amount of radioactivity incorporated into α and β chains after a fixed period of time. This technique is critically dependent on the blood or bone marrow sample being fresh, i.e. less than six hours old. The results are usually expressed as a ratio of $\alpha:\beta$ or $\alpha:\beta+\gamma$. The results of such analysis are shown in Fig. 2.40 and typical globin chain ratios in various conditions are shown in Table 2.4. An abnormal globin chain ratio does not always indicate an abnormality in the rate of synthesis.

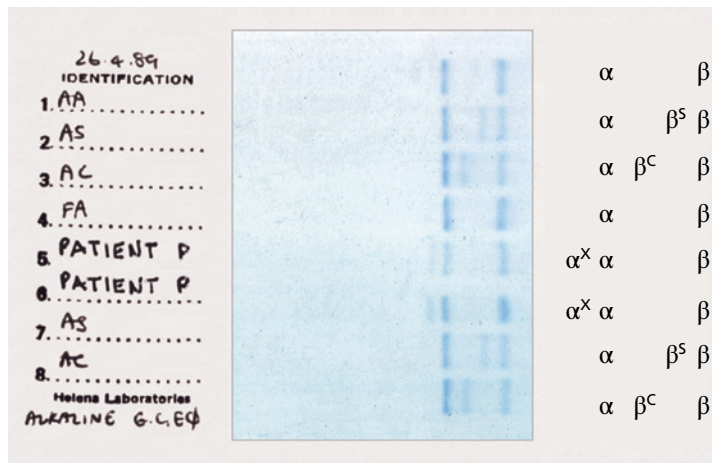


Fig. 2.39 Results of globin chain synthesis analysis showing: (1) α and β chain (normal); (2) α , β^S and β chain (sickle cell trait); (3) α , β^C and β chain (haemoglobin C trait); (4) α and β chain (normal); (5) α^{variant} , α and β chain (identifying an unknown variant as being an α chain variant); (6) α^{variant} , α and β chain (identifying an unknown variant as being an α chain variant); (7) α , β^S and β chain (sickle cell trait); (8) α , β^C and β chain (haemoglobin C trait).

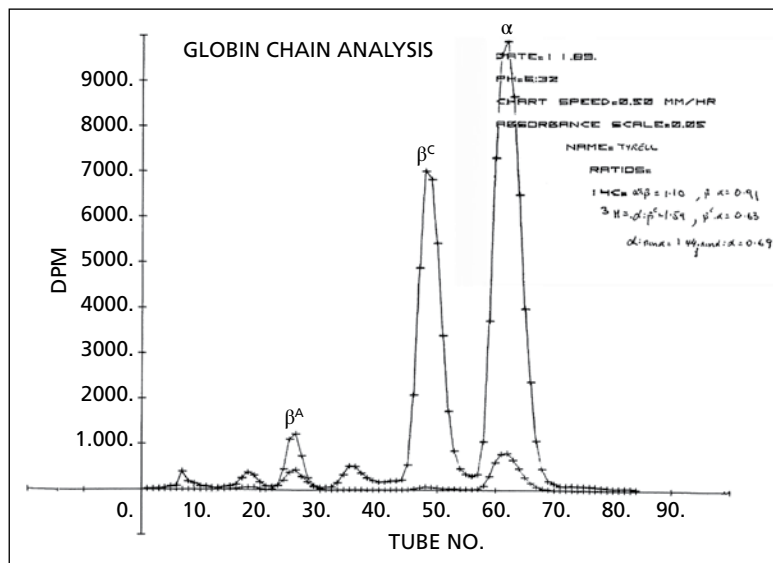


Fig. 2.40 Globin chain synthesis studies in a patient with haemoglobin C/ β^+ thalassaemia compound heterozygosity. (With thanks to Professor Lucio Luzzatto.)

If a globin chain is very unstable the ratio can be abnormal because of very rapid destruction of the unstable chain. Very unstable α chains have been associated with a reduced α :non- α ratio at five minutes but a paradoxically increased ratio at 15 minutes and one hour, which could lead to a misdiagnosis as β thalassaemia intermedia [72]. An abnormal ratio has also sometimes been detected in inherited disorders that do not involve the globin genes; for example, some cases of types I and III congenital dyserythropoietic anaemia have been found to have a

reduced $\alpha:\beta$ ratio, e.g. 0.76 in one case of type III congenital dyserythropoietic anaemia [73].

The more ready availability of molecular techniques for the diagnosis of α thalassaemia has greatly reduced the need for analysis of the rate of α and β globin chain synthesis.

DNA analysis

The most important clinical applications of DNA analysis are: (i) the confirmation of the diagnosis of α^0 thalassaemia trait, particularly

Table 2.4 α : β globin chain synthesis ratio in normal subjects and in various thalassaemias.

Normal or type of thalassaemia	α : β or α :non- α globin chain synthesis ratio
Normal	0.95–1.05
α thalassaemia	
One gene deletion	0.65–0.80
Two gene deletion	0.38–0.60
Haemoglobin H disease	0.20–0.30
β thalassaemia trait (including haemoglobin E trait)	1.67–2.22
β thalassaemia major	No β chain production or 3.6–30

for genetic counselling; (ii) confirmation of the presence of haemoglobin D-Punjab (clinically important) rather than of any other D or G group haemoglobin; and (iii) the prenatal diagnosis of serious disorders of haemoglobin synthesis in the fetus (essential for first trimester diagnosis). In some communities where α , β and δ thalassaemias and complex interactions are relatively common and where silent β thalassaemia occurs, there is a need for more extensive application of DNA analysis to permit accurate diagnosis prior to genetic counselling. In Thailand and Malaysia, where haemoglobin Malay represents around 15% of β thalassaemia alleles, DNA analysis for antenatal diagnosis should include primers for the detection of this variant haemoglobin, since it is silent on electrophoresis and chromatography [74]. For fetal diagnosis, tests are usually carried out on DNA obtained by chorionic villous sampling, a procedure that can be carried out from the 11th week of gestation. Techniques for the analysis of fetal DNA in maternal plasma are gradually being developed although none are currently reliable enough for routine clinical applications.

Some of the techniques that can be applied are shown in Table 2.5 [1, 75–77]. Techniques are modified according to the mutations expected in a given population. For β thalassaemia, the polymerase chain reaction (PCR) is the method of choice when the likely mutations are known. Multiplex PCR and use of techniques such as the detection of the PCR product by an enzyme-linked immunoassay (ELISA) permit automation

of the process [78]. When the likely nature of a mutation is unknown, detection was previously based on linkage analysis using restriction fragment length polymorphisms (RFLP), which requires study of family members with and without the mutation, although this has now been almost entirely replaced by direct sequencing which has become less costly. Increasing numbers of analyses are performed using next-generation sequencing techniques, typically involving a panel of 100 or so relevant genes, which are directly sequenced. Increasing numbers of patients have also had whole genome or whole exome sequencing and bioinformatic analysis of this pre-existing data may yield much of the required diagnostic information [79].

α thalassaemia, including α^0 thalassaemia, can be diagnosed by Southern blot analysis of genomic DNA using α and ζ probes, although this is now used very rarely because it is slow and expensive and usually requires the use of radioactively labelled markers (Fig. 2.41). However, PCR (Fig. 2.42) is cheaper and faster and primers are now available to permit the diagnosis of $-\text{MED}$, $-\text{FIL}$, $-\text{SEA}$, $-\text{THAI}$, $-\alpha^{3.7}$, $-\alpha^{4.2}$, $-(\alpha)^{20.5}$, $\alpha^{\text{NCol}}\alpha$, $\alpha^{\text{HphI}}\alpha$ and also $\alpha\alpha\alpha^{\text{anti3.7}}$ by PCR [80–83]. α thalassaemia can also be diagnosed by a reverse dot blot method [84] in which membrane-bound oligonucleotide allele-specific probes are used as targets for hybridisation of amplified DNA (Fig. 2.43); filter strips are prepared with a probe for the normal α gene and for the variant being sought, permitting the detection of both heterozygotes and homozygotes. Directing sequencing of the α

Table 2.5 Techniques for diagnosis of thalassaemias and haemoglobinopathies by DNA analysis [1, 75–77].

Diagnosis	Test
α thalassaemia	
Deletional α thalassaemia	GAP PCR* MLPA PCR with allele-specific primers (ARMS-PCR)† (including multiplex PCR for deletions common in a specific region) and RQ-PCR [76] Reverse dot blot analysis using mutation-specific probes DNA sequencing‡
Non-deletional α thalassaemia	DNA sequencing
β thalassaemia	
Known common mutations	Sequencing of affected gene DNA sequencing PCR with allele-specific primers Reverse dot blot analysis using mutation-specific probes Gap PCR*
Unknown mutations	Next-generation sequencing DGGE or heteroduplex analysis§ RFLP linkage analysis¶ DNA sequencing
Hb Lepore	Gap PCR DNA sequencing MLPA
$\delta\beta$ thalassaemia	Gap PCR DNA sequencing MLPA
Deletional hereditary persistence of fetal haemoglobin	Gap PCR DNA sequencing MLPA
Hb S	PCR Ddel digestion DNA sequencing
Hb C	PCR with allele-specific primers DNA sequencing‡
Hb E	PCR with allele-specific primers or restriction enzymes DNA sequencing
Hb D-Punjab	PCR, EcoRI digestion DNA sequencing
Hb O-Arab	PCR, EcoRI digestion DNA sequencing

ARMS, amplification refractory mutation system; DGGE, denaturing gradient gel electrophoresis; DNA, deoxyribonucleic acid; MLPA, multiple ligation-dependent probe amplification; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RQ-PCR, real time (quantitative) PCR.

* Gap PCR indicates that there is a ‘gap’ in the DNA sequence (i.e. a deletion) and the primers are chosen so that, if the deletion is present, there is amplification across the ‘gap’. This is useful for the diagnosis of α thalassaemia and the minority of cases of β thalassaemia that result from a relatively large deletion. Other haemoglobinopathies resulting from deletion are also susceptible to detection by this technique.

† ARMS is a PCR technique using two primer sets, one amplifying normal sequences and one abnormal sequences.

‡ DNA sequencing may involve direct sequencing of the whole or part of the affected globin gene, or involve next-generation sequencing, and typically a panel of genes. Next-generation sequencing may be able to detect deletions and other copy number variants, depending on how analysis is performed.

§ DGGE or heteroduplex analysis can be used initially to locate the mutation.

¶ Requires study of the family.

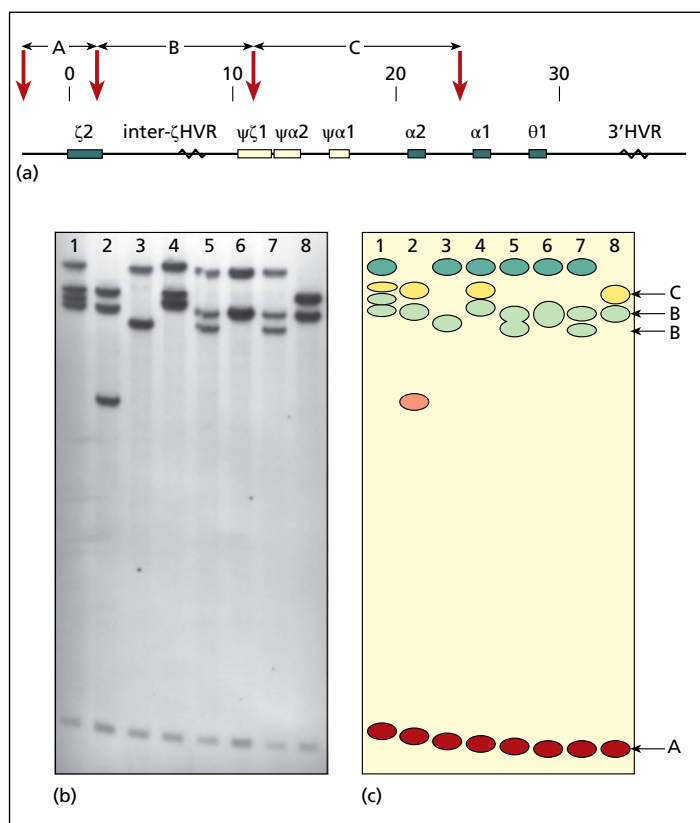


Fig. 2.41 Southern blot analysis following hybridisation of a ζ globin gene probe to *Bgl* II digests of genomic deoxyribonucleic acid (DNA), performed for the diagnosis of α thalassaemia. (a) Diagram of the α globin gene cluster showing sites where *Bgl* II cleaves the DNA (1); *Bgl* II digestion of normal DNA produces three fragments (A, B and C) that will hybridise with the ζ probe: fragment A is a small fragment containing the $\zeta 2$ gene; fragments B and C are larger and each contains part of the $\psi\zeta 1$ gene. Fragment B is of variable length because it contains the inter- ζ hypervariable region (inter- ζ HVR) and for this reason its position on a gel is variable, with two distinct B bands often being present. (b) Gel showing: (lane 1) $\alpha\alpha$ / $-\alpha^{3.7}$; (lane 2) $\alpha\alpha$ / $-\alpha^{4.2}$; (lane 3) $-\alpha^{3.7}$ / $-\alpha^{3.7}$; (lane 4) $\alpha\alpha$ / $-\alpha^{3.7}$; (lane 5) $-\alpha^{3.7}$ / $-\alpha^{3.7}$; (lane 6) $-\alpha^{3.7}$ / $-\alpha^{3.7}$; (lane 7) $-\alpha^{3.7}$ / $-\alpha^{3.7}$; (lane 8) $\alpha\alpha$ / $\alpha\alpha$. (With thanks to Dr Tom Vulliamy.) (c) Explanatory diagram: Lane 8, with normal α genes, shows normal A, B and C fragments; Lanes 1 and 4 show three normal fragments but, in addition, there is a larger fragment that represents an abnormal C fragment consequent on deletion of the 3' *Bgl* II cleavage site by the $-\alpha^{3.7}$ deletion; since fragments of normal size are also present it can be seen that these individuals are heterozygous for this deletion; Lanes 3, 5, 6 and 7 shows loss of the normal C fragment and replacement by a larger C fragment characteristic of $-\alpha^{3.7}$; these individuals are therefore homozygous for $-\alpha^{3.7}$; Lane 2 shows three normal fragments but in addition there is a fragment that is smaller than B and C; this represents a C fragment of reduced size consequent on a $-\alpha^{4.2}$ deletion; as fragments of normal size are also present this individual must be a heterozygote.

globin gene cluster was previously difficult because of the large number of repeated and duplicated sequences and genes in that area, although modern sequencing techniques and bioinformatic analysis have now made this more routine. It is particularly useful for diagnosing non-deletional α thalassaemia.

Other techniques applicable to DNA analysis, including restriction enzyme PCR and high resolution melting analysis (HRMA) for nucleotide variations, and MLPA (multiple ligation-dependent probe amplification) for detection of larger deletions and duplications, are summarised by Vrettou *et al.* [85].

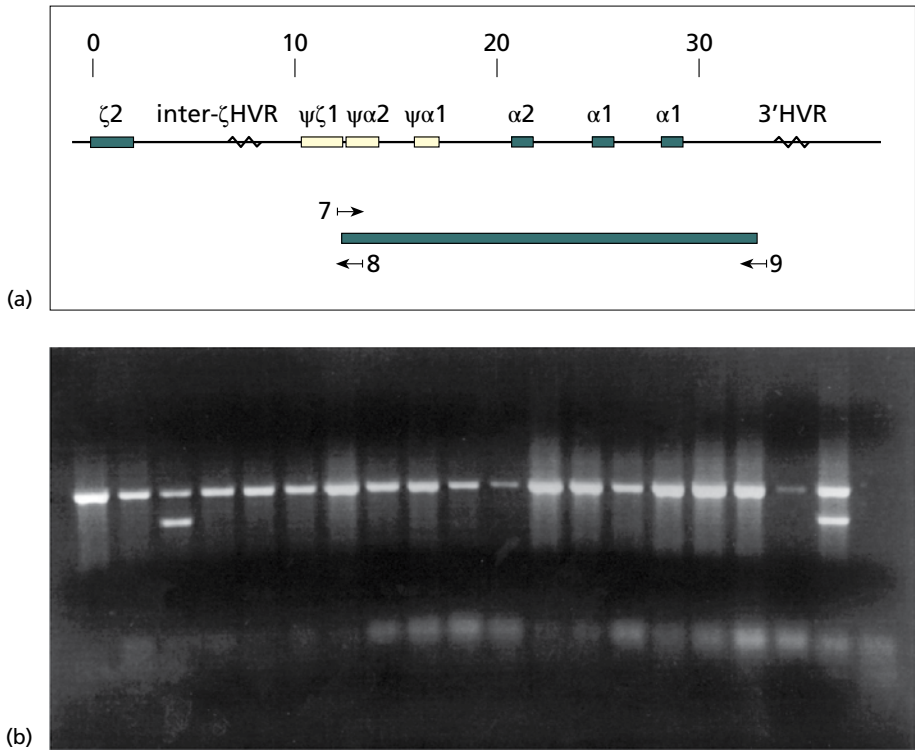


Fig. 2.42 Polymerase chain reaction (PCR) in the diagnosis of α thalassaemia: (a) explanatory diagram adapted from reference [73] showing an α gene cluster and the three primers (7, 8 and 9) described in this paper for the diagnosis of the α^0 thalassaemia determinant, $--^{SEA}$. In a normal genome, primers 7 and 8 amplify a small fragment of DNA but primers 7 and 9 are too widely separated for amplification to occur; in the presence of the large $--^{SEA}$ deletion the sequence to which primer 8 anneals is deleted and the sequences to which primers 7 and 9 bind are brought sufficiently close together that a fragment is amplified; heterozygotes will have two fragments of different sizes whereas a hydropic fetus with $--^{SEA} / --^{SEA}$ will have a single abnormal fragment; (b) gel using the above technique showing a control sample from a subject with $\alpha\alpha / --^{SEA}$ (far right) and 17 individuals being tested, one of whom (third from left) has $\alpha\alpha / --^{SEA}$. (With thanks to Dr Tom Vulliamy.)

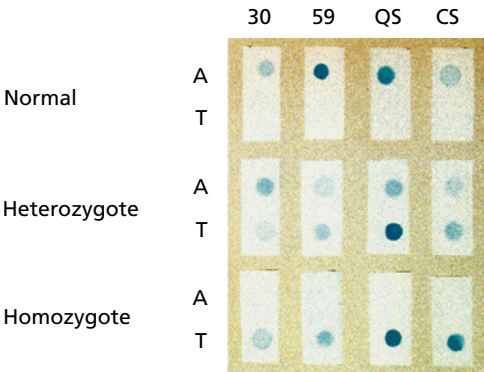


Fig. 2.43 Reverse dot blot analysis for the detection of four non-deletional α thalassaemia determinants, codon 30, codon 59, $\alpha^{Quong\ Size}$ and $\alpha^{Constant\ Spring}$. Amplified DNA samples were hybridised to strips each containing normal (A) and mutant (T) oligonucleotide probes for the particular defect; positive signals appear as blue dots. Samples from heterozygotes show a signal with both the normal and thalassaemia probe whereas samples from homozygotes show a signal only with the thalassaemia probe. (With thanks to Professor V. Chan and the *British Journal of Haematology*.)

Strip assays based on PCR are available for the diagnosis of the most common β thalassaemia mutations in a given geographic area (Mediterranean, India and Middle East, and South-East Asia) and for the diagnosis of triple α and the 20 most common α thalassaemia mutations (www.viennalab.com).

Electrospray ionisation mass spectrometry

Electrospray ionisation mass spectrometry was previously a research technique that was further developed for the identification of variant haemoglobins [86, 87] and is now being applied in a modified form to antenatal and neonatal haemoglobinopathy screening. Variant haemoglobins are identified by the mass difference that the amino acid changes cause, and is particularly useful for identifying variants not associated with charge changes and so more difficult to identify by most conventional techniques. If a mass measurement scan of whole blood is followed by scanning a tryptic digest, giving mass:charge measurement of the peptides produced, variant haemoglobins can be identified fairly specifically [87]. It is possible to determine whether the variant haemoglobin is an α or β chain variant, to estimate the proportion of the variant and to predict the amino acid substitution that could account for any observed change in mass. When there is only a small quantity of a variant haemoglobin present, prior separation by electrophoresis may be needed. Highly unstable haemoglobins may not be identified. The apparatus is very expensive and considerable skill is required in interpretation of results, although the consumable costs are typically very low.

The original technique identified variants on the basis of mass differences and mass: charge analysis of peptides produced by tryptic digestion. The current technique used for screening, which is automated, depends on measurement of the mass: charge ratio using multiple reaction monitoring so that certain specific variants are looked for. Hence, if the common variants, S, C, D-Punjab, E and O-Arab, are targeted, the procedure is useful for antenatal and neonatal screening [88, 89]. The

introduction of instruments more suitable for use in a routine diagnostic laboratory may lead to greater use of the technique.

Quality assurance

All tests should be carried out by appropriately trained personnel following the standard operating procedures (SOP) for each test. A laboratory requires clearly defined protocols for different clinical situations (e.g. for antenatal testing or for the investigation of neonates) (see Chapter 7). All laboratories carrying out haemoglobinopathy testing should participate in an external quality assurance scheme.

Guidelines

Guidelines on selection of tests for specific purposes are available [90, 91].

Appendix

The following methods and kits have been found satisfactory in the laboratories with which the authors are associated.

High performance liquid chromatography

Bio-Rad Variant II

Bio-Rad Laboratories, (UK) Ltd, Watford, Hertfordshire, UK

Capillary electrophoresis

Capillarys 3 Tera

Sebia (UK) Ltd, Camberley, Surrey, UK

Isoelectric focusing

Resolve Hemoglobin Kit and JB-2 staining system

Revvity, Llantrisant, UK

Sickle solubility test

Streck, Sickledex®.

Distributed by Alpha Laboratories, Eastleigh, UK

For other recommended methods, see reference [3].

Check your knowledge

One to five answers may be correct. Answers to almost all questions can either be found in this chapter or can be deduced from information given. Answers are given on page 91.

- 2.1 The following variant haemoglobins have the same mobility as haemoglobin S on cellulose acetate electrophoresis at pH8.4
 - (a) haemoglobin C
 - (b) haemoglobin Lepore
 - (c) haemoglobin D-Punjab
 - (d) haemoglobin G-Philadelphia
 - (e) haemoglobin E
- 2.2 The following haemoglobins have the same mobility as haemoglobin A on agarose gel electrophoresis at pH6.2
 - (a) haemoglobin E
 - (b) haemoglobin S
 - (c) haemoglobin D-Punjab
 - (d) haemoglobin C
 - (e) haemoglobin G-Philadelphia
- 2.3 Satisfactory methods for the precise quantification of the haemoglobin A₂ percentage include
 - (a) scanning densitometry
 - (b) HPLC
 - (c) isopropanol test
 - (d) capillary electrophoresis
 - (e) cellulose acetate electrophoresis followed by elution
- 2.4 The following variant haemoglobins have the same mobility as haemoglobin C on cellulose acetate electrophoresis at pH8.4
 - (a) haemoglobin A₂
 - (b) haemoglobin Lepore
 - (c) haemoglobin O-Arab
 - (d) haemoglobin C-Harlem
 - (e) haemoglobin E
- 2.5 A false positive sickle solubility test may be caused by
 - (a) the presence of haemoglobin C
 - (b) anaemia
 - (c) increased plasma proteins
 - (d) the presence of haemoglobin D
 - (e) the presence of numerous Heinz bodies
- 2.6 If an unstable haemoglobin is suspected relevant tests include
 - (a) blood film
 - (b) reticulocyte count
 - (c) isopropanol test
 - (d) heat stability test
 - (e) alkali denaturation
- 2.7 Techniques applicable in the diagnosis of α or β thalassaemia in a fetus include
 - (a) reverse dot blot analysis
 - (b) PCR
 - (c) isopropanol test
 - (d) direct sequencing of the α or β globin gene
 - (e) microcolumn chromatography
- 2.8 Quantification of haemoglobin A₂ can be inaccurate in the presence of
 - (a) a raised haemoglobin F
 - (b) diabetes mellitus
 - (c) haemoglobin D-Punjab
 - (d) haemoglobin E
 - (e) haemoglobin S
- 2.9 The presence of haemoglobin S may be conclusively demonstrated by
 - (a) a positive sickle test
 - (b) a positive sickle solubility test
 - (c) an immunoassay
 - (d) PCR with appropriate restriction enzymes
 - (e) electrophoresis on cellulose acetate at pH8.3
- 2.10 A positive test for an unstable haemoglobin can result from
 - (a) an aged sample
 - (b) the presence of a high concentration of haemoglobin F
 - (c) α thalassaemia trait
 - (d) β thalassaemia trait
 - (e) the presence of an unstable haemoglobin
- 2.11 An α : β chain synthesis ratio of 0.25:1 is compatible with
 - (a) α thalassaemia trait
 - (b) normal
 - (c) β thalassaemia trait
 - (d) haemoglobin H disease
 - (e) β thalassaemia major

Further reading

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Answers to questions

- | | | | |
|---|---|---|--|
| 2.1 (a) F
(b) T
(c) T
(d) T
(e) F | 2.4 (a) T
(b) F
(c) T
(d) T
(e) T | 2.7 (a) T
(b) T
(c) F
(d) T
(e) F | 2.10 (a) T
(b) T
(c) F
(d) F
(e) T |
| 2.2 (a) T
(b) F
(c) T
(d) F
(e) T | 2.5 (a) F
(b) F
(c) T
(d) F
(e) T | 2.8 (a) F
(b) F
(c) T
(d) T
(e) T | 2.11 (a) F
(b) F
(c) F
(d) T
(e) F |
| 2.3 (a) F
(b) T
(c) F
(d) T
(e) T | 2.6 (a) T
(b) T
(c) T
(d) T
(e) F | 2.9 (a) T
(b) F
(c) T
(d) T
(e) F | |

3 α , β , δ and γ thalassaemias and related conditions

Thalassaemia is the name given to a globin gene disorder that results in a diminished rate of synthesis of one or more of the globin chains and consequently a reduced rate of synthesis of the haemoglobin or haemoglobins of which that chain constitutes a part and an excess of unpaired globin chains from the unaffected genes. The condition was first described by Cooley and Lee in 1925 [1] with the name 'thalassaemia' from the Greek *θαλασσαια*, sea, via 'thalassic anaemia', being given by Whipple and Bradford in 1936 [2]. In the late 1930s the hereditary nature of thalassaemia was clearly identified in both Greece and Italy. It is probable that the failure to identify thalassaemia as a discrete entity in the Mediterranean area until after its description in the United States was because malaria, as a cause of childhood anaemia and splenomegaly, was still prevalent around the Mediterranean.

In thalassaemia, a significantly reduced rate of synthesis of one type of globin chain leads to unbalanced chain synthesis with excess of the unaffected globin chain contributing to the pathological effects, causing either damage to erythroid precursors and ineffective erythropoiesis or damage to mature erythrocytes and haemolytic anaemia, or both. Thalassaemia can be classified according to the phenotype or the genotype. The major types of thalassaemia, classified according to the genotype, are shown in Table 3.1. Thalassaemia can result from deletion of a large part or all of a gene (as is usual in α thalassaemia) or from a small deletion or other mutation of a gene (as is usual in β thalassaemia). Mutations of α and β genes are of potential clinical significance since there is a reduced rate of synthesis of haemoglobin A, the

major haemoglobin of adult life. A significant clinical disorder usually results only when both of the β genes or either three or four of the α genes are affected. γ thalassaemia would only be of potential significance in intrauterine and early neonatal life when haemoglobin F is the major haemoglobin. However, since there are four γ genes, significant disease is unlikely. δ thalassaemia is of no clinical significance except that its presence may interfere with the diagnosis of coexisting β thalassaemia.

Thalassaemia usually results from mutation of a single globin gene or from deletion of one or more globin genes. One mechanism of deletion is unequal crossover between chromosomes at meiosis so that parts of two genes are deleted and the 5' end of one gene fuses with the 3' end of another gene. This is the mechanism underlying some types of α thalassaemia trait (Fig. 3.1). Another defect of this type leads to deletion of part of both a δ gene and a β gene with production of a $\delta\beta$ fusion gene, leading to synthesis of haemoglobin Lepore (named from the Italian family in whom it was first described) (see Fig. 1.14a). The rate of synthesis of the abnormal $\delta\beta$ chain is slower than the rate of synthesis of β chain so that haematological features are very similar to those of β thalassaemia. Since one δ gene has effectively been lost there is also a reduced rate of synthesis of δ chain and a reduced proportion of haemoglobin A₂. A thalassaemic phenotype can also result from a mutation that leads to formation of a very unstable haemoglobin molecule or a very unstable globin chain that precipitates before it is incorporated into haemoglobin.

Table 3.1 Classification of the thalassaemias.

Type of thalassaemia	Chain or chains synthesised at a reduced rate	Haemoglobin or haemoglobins synthesised at a reduced rate
Alpha: α^0 or α^+	α	A, A ₂ and F
Beta: β^0 or β^+	β	A
Gamma: γ	G γ and/or A γ	F
Delta: δ^0 or δ^+	δ	A ₂
Delta beta: $\delta\beta^0$ or $\delta\beta^+$	δ and β	A and A ₂
Λ gamma delta beta: $\Lambda\gamma\delta\beta^0$	$\Lambda\gamma$, δ and β	A and A ₂
Epsilon gamma delta beta*: $\epsilon^G\gamma\Lambda\gamma\delta\beta^0$	ϵ , G γ , A γ , δ and β	A, A ₂ and F*
Haemoglobin Lepore	δ and β	A and A ₂ †

* Often referred to as $\gamma\delta\beta$ thalassaemia; in fetal life there is decreased synthesis of haemoglobins Gower 1, Gower 2 and Portland 1.
† Haemoglobin Lepore is synthesised at a reduced rate in comparison with haemoglobin A but at an increased rate in comparison with haemoglobin A₂.

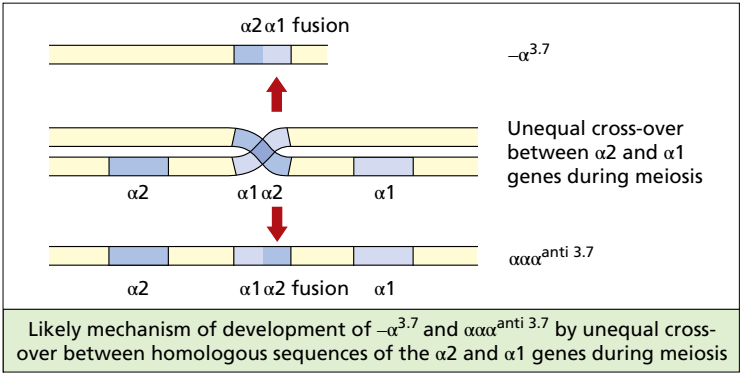


Fig. 3.1 Diagram showing the likely mechanism for the occurrence of the α gene deletion ($-\alpha^{3.7}$) and triple α ($\alpha\alpha\alpha^{anti 3.7}$) by unequal crossover between homologous sequences of the $\alpha 2$ and $\alpha 1$ genes during meiosis.

Unbalanced globin chain synthesis can also result from duplication of a globin gene, leading to an increased rate of synthesis of that globin chain. Thus there may be three, four or even five alpha genes on a single chromosome (instead of the normal two), referred to as triple alpha, quadruple alpha and quintuple alpha respectively. Duplication of a gene is not usually of any clinical significance but when it coexists with thalassaemia it may either lessen the chain imbalance and ameliorate the condition or aggravate the chain imbalance and thus increase the severity of the disorder. Thus if one chromosome has $\alpha\alpha\alpha$ and the other has $-\alpha$ there is likely to be negligible chain imbalance and no haematological effect. However, the coexistence of $\alpha\alpha\alpha/\alpha\alpha$ or $\alpha\alpha\alpha/\alpha\alpha\alpha$ and β thalassaemia increases the severity of the β thalassaemia due to the increased excess of unpaired α globin chains.

Changes in the numbers of genes, either through large deletions or duplications, are referred to as copy number variants (CNVs) and particularly occur in areas of the genome with repetitive sequences of DNA, such as the α globin locus (with duplicated α and ζ globin genes) and to a lesser extent the β globin locus (with duplicated γ globin genes).

α thalassaemias

The alpha or α thalassaemias are a group of conditions resulting from a reduced rate of synthesis of α globin. There are more than 140 genetic defects underlying α thalassaemia. The severity of the defect is very variable. At one extreme is a completely asymptomatic condition, resulting from deletion or dysfunction of one of the four α genes, which

produces either a trivial abnormality in the blood count and film or no abnormality at all. At the other extreme is haemoglobin Bart's hydrops fetalis, a condition that is generally incompatible with life, usually resulting from deletion of all four α genes and consequent total lack of α globin synthesis. Some of the clinicopathological features of α thalassaemia syndromes result from the lack of α globin chain while others result from damage to red cell precursors and mature red cells by excess non- α chains. Excess β and γ chains can, in the absence of sufficient α chain, form haemoglobins with β or γ chain tetramers. The resultant haemoglobins are haemoglobin H with β tetramers, first described by Rigas and colleagues in 1955 [3], and haemoglobin Bart's with γ tetramers, first described by Fessas and Papaspyrou in 1957 [4]. Haemoglobin H was so named because haemoglobin G (now known as haemoglobin Korle Bu) had been described a year earlier [5]. The current name of haemoglobin Bart's, previously designated haemoglobin Fessas and Papaspyrou or haemoglobin F and P, was given by Ager and Lehmann in 1958 [6], since the patient in whom they observed it was a patient of St Bartholomew's Hospital in London.

When both α genes on a single chromosome are deleted or transcriptionally completely inactive, the designation α^0 thalassaemia (α zero thalassaemia) is used. When there is reduced but detectable production of α globin chain, for example when only one of the two α genes on a chromosome is deleted, the

designation α^+ thalassaemia (α plus thalassaemia) is used. Function of the α globin gene cluster at 16p13.3 requires the presence of a major upstream regulatory element, the locus control region alpha (LCRA), previously referred to as HS -40 because it is 40kb upstream of the $\zeta 2$ locus; thalassaemia can occur with completely normal α genes if the locus control region is deleted.

α thalassaemia can be divided broadly into deletional and non-deletional thalassaemia. Deletional α thalassaemia results in either α^0 or α^+ thalassaemia, depending on the length and nature of the deletion. Non-deletional α thalassaemia usually leads to α^+ thalassaemia trait. Non-deletional α thalassaemias can result from variants of the $\alpha 2$ gene ($\alpha^T \alpha$ thalassaemia) or the $\alpha 1$ gene ($\alpha \alpha^T$ thalassaemia). More than 70 such mutations have been reported but most of these are rare. Recognised cases of non-deletional thalassaemia are much more often caused by mutation of the $\alpha 2$ gene (*HBA2*) than of the $\alpha 1$ gene (*HBA1*). This is likely to be, at least in part, because the $\alpha 2$ gene is more highly expressed than the $\alpha 1$ gene. Non-deletional variants in the $\alpha 2$ gene have a more severe phenotype than deletion of the $\alpha 2$ gene as upregulation of the $\alpha 1$ gene occurs to a greater extent in the latter instance.

Important deletions that lead to α thalassaemia are shown diagrammatically in Fig. 3.2 and deletions and mutations are summarised in Tables 3.2 and 3.3 [7–24]. Deletions and non-deletional variants causing α thalassaemia fall into nine broad categories.

Fig. 3.2 Diagrammatic representation of some common deletions that can lead to α^0 and α^+ thalassaemia; the shaded blocks indicate the length of the deletion.

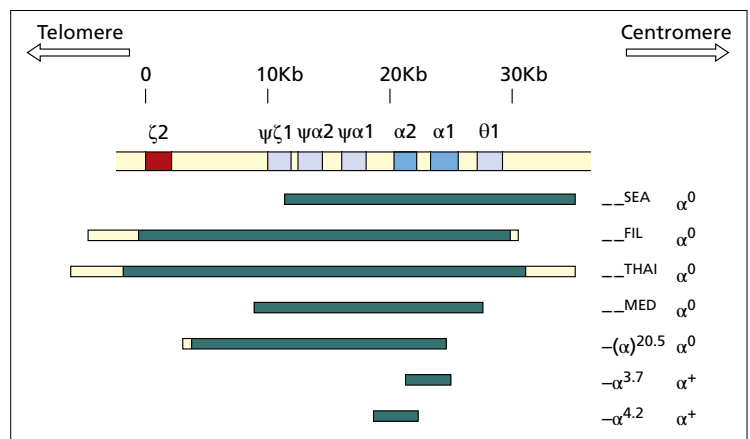


Table 3.2 Classification of deletional α thalassaemia.

Type of deletion	Phenotype	Number of examples recognised*	Examples
Deletion involving one or both α genes			
Deletion of all or part of one α gene	α^+ thalassaemia,	At least 7	$-\alpha^{4.2}$, $-\alpha^{3.7I}$, $-\alpha^{3.7II}$, $-\alpha^{3.7III}$, $-\alpha^{3.5}$, $\alpha(\alpha)^{5.3\ddagger}$, $-\alpha^{2.7}$
Deletion of all or part of both α genes but without deletion of <i>LCRA</i>	α^0 thalassaemia	At least 20	$-\alpha^{SEA}$, $-\alpha^{THAI}$, $-\alpha^{MED}$, $-\alpha^{FIL}$, $-\alpha^{BRIT}$, $-\alpha^{SPAN}$, $-\alpha^{YEM}$, $-(\alpha)^{20.5\ddagger}$, $-(\alpha)^{5.2\ddagger}$,
Deletion of both α genes and of <i>LCRA</i> (100–>250 kb)	α^0 thalassaemia	At least 8, without other phenotypic abnormality	$-\alpha^{DUTCHII}$
Extensive loss of 16p13.3 (1–2 Mb) including both α genes and <i>LCRA</i> †	α^0 thalassaemia	At least 17, with mental retardation and dysmorphism	$-\alpha^{BO}$
Deletion of $\alpha 1$ gene and 18–20 kb downstream of $\alpha 1$ gene [17]	α^0 thalassaemia	At least 1	$(\alpha)^{-ZF} \ddagger$
Deletion leaving α genes intact			
Deletion of upstream major regulatory element (<i>LCRA</i> , MCS-R2) without deletion of α genes [24]	α^0 or very severe α^+ thalassaemia	At least 12	$(\alpha\alpha)^{RA}\S$, $(\alpha\alpha)^{TAT}\S$, $(\alpha\alpha)^{MM}\S$, $(\alpha\alpha)^{IJ}\S$, $(\alpha\alpha)^{KS}\S$

* Likely to be an underestimate.

† (α) indicates that the gene is present but non-functional.

‡ Loss of 16p13.3 may be the result of deletion, inversion plus deletion, formation of a ring chromosome 16 that lacks the α gene cluster or unbalanced inheritance of a derivative (16) lacking 16p13.3 from a parent who had a balanced translocation, e.g. t(1;16), t(5;16) or t(16;20) [19].

§ $(\alpha\alpha)$ indicates that both α genes are present but non-functional.

Deletional

1 Deletion of all or part of one or both α genes:

- deletion of an $\alpha 2$ gene (α^+ thalassaemia) (e.g. $-\alpha^{4.2}$);
- deletion of an $\alpha 1$ gene (α^+ thalassaemia);
- deletion of part of $\alpha 2$ gene and part of $\alpha 1$ gene with formation of a fusion α gene (α^+ thalassaemia) (e.g. $-\alpha^{3.7}$);
- deletion of an $\alpha 1$ gene and more than 18 kb downstream but with inactivation of the remaining structurally normal $\alpha 2$ gene by a negative positional effect (α^{-ZF}) [7, 17] (α^0 thalassaemia);
- deletion of adjacent $\alpha 2$ and $\alpha 1$ genes (α^0 thalassaemia);
- deletion of both α genes and *LCRA* (α^0 thalassaemia);
- deletion of the MCS-R2 enhancer of the α genes.

There are at least 36 different deletions known, some also having deletion of the ζ gene and intervening sequences; this category includes extensive deletions or unbalanced translocations resulting in loss of the telomere of chromosome 16 causing a syndrome of α thalassaemia trait (α^0 thalassaemia), dysmorphism and mild to moderate intellectual disability referred to as the ATR-16 syndrome (Table 3.2). The two most common α^+ mutations, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, occasionally coexist with triple α on the same chromosome, designated HK $\alpha\alpha$ ($-\alpha^{3.7}$ and $\alpha\alpha\alpha^{\text{anti-4.2}}$) and anti-HK $\alpha\alpha$ ($-\alpha^{4.2}$ and $\alpha\alpha\alpha^{\text{anti-3.7}}$) [25]; unlike the simple heterozygotes for $-\alpha^{3.7}$ and $-\alpha^{4.2}$, there is no risk of haemoglobin H disease in offspring.

2 Deletion of the upstream major regulatory element including the *LCRA* with marked downregulation or abrogation of expression

Table 3.3 Classification of non-deletional α thalassaemia.

Type of mutation	Phenotype	Number of examples recognised	Examples
RNA splice site mutation in $\alpha 1$ or $\alpha 2$ gene (donor or acceptor site)	α^+ thalassaemia	At least 3 ($\alpha 2$ donor site, $\alpha 2$ acceptor site, $\alpha 1$ acceptor site)	<i>HBA2</i> IVS1 (-5nt) donor splice site mutation in Mediterranean area and Middle East
RNA polyadenylation signal mutations	$\alpha^+-\alpha^0$ thalassaemia (i.e. severe α^+) or α^+	At least 5 (described only for $\alpha 2$ gene which is likely to account for the severe phenotype)	<i>HBA2</i> AATAAA \rightarrow AATAAG ($\alpha^{\text{PA}1}\alpha$, also known as $\alpha^{\text{TSaudi}}\alpha$) <i>HBA2</i> AATAAA \rightarrow AATA-- ($\alpha^{\text{T India}}\alpha$, India and Thailand)
Impaired RNA translation consequent on initiation codon or initiation consensus sequence mutation	α^+ thalassaemia, $\alpha^+-\alpha^0$ or, when the mutation occurs in association with deletional α thalassaemia, α^0 thalassaemia	At least 5 (two in $\alpha 2$ gene, one in $\alpha 1$ gene, two in single α gene)	<i>HBA2</i> ATG \rightarrow ACG, GTG or A-G; $-\alpha^{3,7}$ ATG \rightarrow GTG (mutation in association with deletion gives α^0 phenotype)
Impaired RNA translation consequent on a frameshift or nonsense mutation	α^+ or α^0 thalassaemia	At least 5 (4 frameshift plus 1 nonsense)	Codon 30/31 (-4nt) frameshift and $\alpha 2$ CD116 GAG \rightarrow TAG nonsense mutation
Impaired RNA translation consequent on a termination codon mutation leading to an elongated mRNA and α globin chain	α^+ thalassaemia	At least 5 (all $\alpha 2$ gene)	Hb Constant Spring TAA \rightarrow CAA ($\alpha^{\text{CS}}\alpha$), Hb Icaria TAA \rightarrow AAA ($\alpha^{\text{Ic}}\alpha$), Hb Koya Dora TAA \rightarrow TCA, Hb Seal Rock TAA \rightarrow GAA, Hb Paksé TAA \rightarrow TAT
Production of highly unstable α chain as a result of point mutation or a small deletion	α^+ thalassaemia	At least 18: 14 point mutations, 4 small deletions; 11 affecting $\alpha 2$ gene, 4 affecting $\alpha 1$ gene and 3 affecting a single α gene	Hb Agrinio ($\alpha^{\text{Agr}}\alpha$), Hb Petah Tikvah ($\alpha^{\text{PT}}\alpha$), Hb Quong Sze ($\alpha^{\text{QS}}\alpha$), Hb Suan Dok ($\alpha^{\text{SD}}\alpha$), and Hb Evaston (point mutations); Hb Taybe (small deletion); Hb Adana ($\alpha^{\text{Adana}}\alpha$ or $\alpha\alpha^{\text{Adana}}$)
Lack of a transactivating factor encoded by the <i>ATRX</i> gene	α^+ thalassaemia		ATR-X syndrome

of both structurally normal α genes [26] (it is estimated that α chain production is less than 1% of normal so this is effectively α^0 thalassaemia; there are at least 12 examples) (Table 3.2).

Non-deletional

3 Mutations affecting ribonucleic acid (RNA) splicing (Table 3.3). The mutation IVS1 117 G \rightarrow A in the $\alpha 1$ gene, for example, is an acceptor splice site mutation, found in India, that makes

the gene non-functional; splice site variants such as this, since they affect only one of the two α genes, produce an α^+ phenotype.

4 Variants affecting polyadenylation (Table 3.3). The mutation $\alpha^{\text{TSaudi}}\alpha$ ($\alpha^{\text{PA6 A}}\alpha$), for example, which is common around the Mediterranean, is one of a number of variants affecting the highly conserved messenger RNA (mRNA) cleavage and polyadenylation (PA) signal; this mutation leads to a marked reduction in α chain synthesis,

giving a severe α^+ phenotype which is sometimes designated $\alpha^+-\alpha^0$; other variants affecting polyadenylation may be less severe.

5 Variants affecting RNA translation (Table 3.3):

- initiation codon or initiation consensus sequence variants leading to absent or reduced translation;
- frameshift variants resulting from small deletions or deletion plus insertion or nonsense mutations acting as premature termination codons, leading to inactivation of the gene or synthesis of a very unstable α chain;
- mutation of a termination codon to a coding sequence leading to an elongated α chain that is synthesised at a reduced rate, possibly because of instability of the mRNA; examples of mutations of the termination codon leading to an elongated α chain include haemoglobin Constant Spring (found in southern China, Thailand, Cambodia, Vietnam, Laos and around the Mediterranean, e.g. Greece and Sicily), haemoglobin Koya Dora (found with a 10% prevalence in the Koya Dora tribe in Andhra Pradesh, India), haemoglobin Icaria, haemoglobin Seal Rock and haemoglobin Paksé (found in Laos, Cambodia and Thailand); each of these α chains is elongated by 31 amino acids as translation continues into the 3' untranslated region (UTR) until a downstream termination codon is encountered within the polyadenylation signal sequence.

6 Variants causing marked post-translational instability of a highly abnormal α chain, usually as the result of a defect in the haem pocket, in $\alpha\beta_1$ contacts or affecting binding to α -haemoglobin stabilising protein (which is necessary for folding and solubility of free α chains) [10, 18, 27]; examples include haemoglobin Quong Sze ($\alpha^{125 \text{ Leu} \rightarrow \text{Pro}}\alpha$ or $\alpha^{\text{QS}}\alpha$), which is found in Kurdish Jews and in South-East Asia, haemoglobin Agrinio ($\alpha^{29 \text{ Leu} \rightarrow \text{Pro}}\alpha$ or $\alpha^{\text{Agr}}\alpha$), found in the Mediterranean area (Greece and Cyprus) and in South-East Asia, and haemoglobin Adana, found in Indonesia and as occasional cases in other parts of the world.

7 Deletion that leads to loss of the $\alpha 1$ gene and truncation of a downstream gene, *LUC7L*, which is transcribed in the opposite direction, leading to transcription of RNA that is antisense with regard to the $\alpha 2$ gene, in turn leading to methylation and silencing of the remaining $\alpha 2$ gene [28].

8 Gain-of-function mutation between the α genes and their locus control region that creates a promoter-like region that interferes with transcription of both α genes leading to $(\alpha\alpha)^T$ [29].

9 Transactivating abnormality resulting from mutation in the *ATRX* gene at Xq21.1 (previously known as the XH2 locus), which encodes a deoxyribonucleic acid (DNA) helicase [30], resulting in a syndrome of moderately severe intellectual disability, dysmorphism and α thalassaemia in males, referred to as the *ATRX* syndrome; more than 180 families have been described [18].

It is likely that the two most common deletions, $-\alpha^{3,7}$ and $-\alpha^{4,2}$ are both the result of an unequal crossover during meiosis with the result that when this mutation first arose, one chromosome was left with a single α gene while the other had a triplicated α gene. In the case of $-\alpha^{3,7}$ the single α gene and the central gene of the three α genes is a fusion gene (see Fig. 3.1). Either abnormal chromosome could have passed into the gamete and thus the fetus and thus both $\alpha\alpha\alpha^{\text{anti}3,7}$ and $\alpha\alpha\alpha^{\text{anti}4,2}$ are known to exist. It appears that both these mutational events occurred a number of times in a variety of ethnic groups. Further investigation of $-\alpha^{3,7}$ has established that there are in fact three slightly different deletions, occurring in different ethnic groups, which are designated $-\alpha^{3,7\text{I}}$, $-\alpha^{3,7\text{II}}$ and $-\alpha^{3,7\text{III}}$. Only $-\alpha^{3,7\text{I}}$ is common in many ethnic groups. $-\alpha^{3,7\text{II}}$ has been described in India and Nepal whereas $-\alpha^{3,7\text{III}}$ is confined to Oceania [31]. Deletional α^+ thalassaemia, mostly $-\alpha^{3,7}$, is one of the most common disease-causing variants in the world because it offers some protection against malaria, whereas triplicated α globin alleles are relatively uncommon, presumably because they offer no significant survival advantage.

A variant haemoglobin may be predicted from the DNA sequence in patients with thalassaemia but may be undetectable (e.g. haemoglobin Quong Sze), or present in very low amounts (e.g. haemoglobin Suan Dok), because of very marked instability of the α chain, the $\alpha\beta$ dimer or the haemoglobin molecule. Sometimes a hyperunstable haemoglobin is detectable only after splenectomy. When the haematological features are those of α thalassaemia rather than of an unstable haemoglobin, classification as non-deletional α thalassaemia is appropriate. When an α chain variant is only moderately unstable it will constitute a larger proportion of

total haemoglobin and will produce the phenotype of a Heinz body haemolytic anaemia and classification as an unstable haemoglobin is then appropriate. An unstable haemoglobin can interact with α^0 determinants to cause haemoglobin H disease (see Table 3.6).

The types of mutation most commonly found in different ethnic groups are shown in Table 3.4 and the incidence of α^0 and α^+ thalassaemia in different ethnic groups in Table 3.5 [16, 32–76]. Further data are available in reference [76]. Overall, the highest prevalence of α thalassaemia

Table 3.4 Types of mutation most often responsible for α thalassaemia in different ethnic groups.

Ethnic group	Type of thalassaemia	Designation	Nature of mutation
South-East Asia and southern China	α^0	-- _{SEA}	Deletion of both α genes
		-- _{FIL}	Deletion of both α genes
		-- _{THAI}	Deletion of both α genes
	α^+	$-\alpha^{4.2}$	Deletion of $\alpha 2$ gene
		$-\alpha^{3.7}$	Deletion of part of both α genes with formation of an $\alpha 2\alpha 1$ fusion gene
		$\alpha^{\text{CS}}\alpha$	Haemoglobin Constant Spring, reduced rate of synthesis of a haemoglobin with an elongated α chain
		$\alpha^{\text{NcoI}}\alpha$	Mutation in initiation codon of $\alpha 2$ gene
		$\alpha\alpha^{\text{NcoI}}$	Mutation in initiation codon of $\alpha 1$ gene
		$\alpha^{\text{Suan Dok}}\alpha$	Very unstable α chain
		$\alpha^{\text{Quong Sze}}\alpha$	Very unstable α chain
Mediterranean (particularly Greece and Cyprus)	α^0	-- _{MED I} , -- _{MED II}	Deletion of both α genes
		$-(\alpha)^{20.5}$	Deletion of all of one α gene and part of the other
	α^+	$-\alpha^{3.7}$	As above
		$\alpha^{\text{TSaudi}}\alpha$	Polyadenylation signal sequence mutation
Middle East	α^+	$\alpha^{\text{Hph}}\alpha$	Small frameshift mutation of IVS1 donor site
		$\alpha^{\text{TSaudi}}\alpha$	Polyadenylation signal sequence mutation
India	α^+	-- _{MED}	Deletion of both α genes
		$-\alpha^{3.7}$	As above
		$-\alpha^{4.2}$ (less common than $-\alpha^{3.7}$)	As above
		$\alpha\alpha^{\text{IVS1 nt 117 G} \rightarrow \text{A}}$	Acceptor splice site mutation
Sri Lanka	α^+	$\alpha^{\text{Koya Dora}}\alpha$	Mutation of termination codon resulting in an extended unstable α chain
		$-\alpha^{3.7}$	As above
Nepal	α^+	$-\alpha^{4.2}$	As above
		$-\alpha^{3.7}$	As above
Africa, African-American and African-Caribbean	α^+	$-\alpha^{3.7}$	As above
		$-\alpha^{3.7}$	As above
Melanesia	α^+	$-\alpha^{3.7}$	As above
		$-\alpha^{4.2}$ (less frequent than $-\alpha^{3.7}$ except in Papua New Guinea)	As above
Polynesia	α^+	$-\alpha^{3.7}$	As above; $-\alpha^{3.7\text{III}}$ is most characteristic of Polynesia and almost confined to this area

Table 3.5 The prevalence of α^0 , α^+ and β thalassaemia heterozygosity in different countries and ethnic groups (derived from multiple sources including references [16, 32–76]).

Country or ethnic group	α^0 thalassaemia	α^+ thalassaemia	β thalassaemia
Greece	About 1.5%	7–10%	6–28% (overall 8%)
Cyprus	Uncommon, averaging around 2% (both Greek and Turkish Cypriots)	26% (including 1% $\alpha^T\alpha$)	14–18% (incidence is similar in Greek and Turkish Cypriots, overall 15%)
Turkey	Uncommon (about 0.6%)	6%	1–37% (high incidence confined to Eti-Turks on south-eastern coast, overall 2–3%)
Italy	Rare (0.5% in Sardinia, 0.2% in Sicily, even lower in southern Italy)	10% in Sicily, 28% in Sardinia	1–30% (overall 4%) (highest prevalence in Po delta, Sardinia (10%), southern Italy and Sicily)
Spain	Very rare (0.2%)	2%	1–8% (highest in Minorca, southern Spain and Galicia); 5% in Spanish gypsies
Portugal	Rare	10%	0.5–7.5%
France – Corsica			3%
Malta			3–4% [60]
Eastern Europe (Romania, Bulgaria, Former Yugoslavia, former Soviet Union)	0.05% in North Macedonia (former Yugoslavia) [61]	1.5% in North Macedonia (former Yugoslavia) [61]	Overall 2–20% (rare in ex-Czechoslovakia, Hungary, northern former USSR; higher in Romania, 3% in Uzbekistan, 5% in Tajikistan, 5.5% in Azerbaijan); 1–10% (average 2.6%) in North Macedonia (former Yugoslavia) [61]; 0.5–19.9% (average 2.5%) in Bulgaria [62], 8.4% in one study in Albania [63]
British (white)	Rare (occurs particularly in Lancashire and Cheshire; 0.05% in Wigan, UK)	<1%	<0.5%, probably about 0.1%
Middle East (Iran, Iraq, Syria, Lebanon, Jordan, Bahrain, United Arab Emirates, Saudi Arabia, Israel, Palestine, Yemen)	Rare (occurs rarely in Israel, United Arab Emirates, Iran; $-\alpha^{YEM}/$ is occasionally found in Yemenite Jews and $-\alpha^{MED}/$ in Middle Eastern Jews; occasional $-\alpha^{MED}/$ in Arabs; 6% of α thalassaemia alleles in Iran were $-\alpha^{MED}$ [64])	<1–20% (overall 9%); 47% in Saudi Arabia, highest in eastern province; 18–50% in United Arab Emirates, 64% in Kuwait, 89% in Oman, relatively frequent in Israeli Arabs and Yemenite Jews, 9% in Ashkenazi Jews	Overall 2–20%: Iran 1–7% (overall 3%), Iraq 2–7% (4% in North-East Iraq [65], 7% in Erbil province [66]), Syria 1%, Lebanon 2–6%, Jordan < 1–4%, Bahrain 2–3%, Oman 1–2%, United Arab Emirates 8% [67], Central Saudi Arabia 3–4%, Eastern Saudi Arabia 13–18%, overall Saudi Arabia 1–1.8% [68], Yemen 1–2%, Palestine (Gaza strip) 4%, Israeli Arabs 3–25%, Yemenite Jews 9%, Kurdish Jews 20%, Ashkenazi Israeli Jews <0.1%, other Middle Eastern Jews 2–4%

Table 3.5 *Continued.*

Country or ethnic group	α^0 thalassaemia	α^+ thalassaemia	β thalassaemia
North Africa and Horn of Africa (Morocco, Tunisia, Algeria, Libya, Egypt, Sudan, Ethiopia)	0.6% α^0 in Algeria ($-\alpha^{\text{MED}}$ and $-\alpha^{\text{20.5}}$ [69]; not detected in Tunisia [70]; 5% $-\alpha/\alpha$ or haemoglobin H disease in Egypt [71]	5–8% overall; 4% in Algeria [69]; 2–4% in Tunisia [70]; 10% in Egypt [71]	Libya <1–11%, Algeria <1–15% (overall 2%), Morocco <1–7% (overall 3%), Tunisia 3.5%, Egypt <1–9%, Sudan 1–10% (overall 4%), Ethiopia <1–8%
West Africa	Very rare	Gambia 8–15%, Togo 46%, Nigeria 8–58%, Senegal 22%, Benin and Burkina Faso 29%, Ivory Coast 39%	Overall 1–14%, Senegal <1–5%, Liberia <1–9%, Ivory Coast 1–12%, Mali <1%, Burkina Faso 2–12%, Ghana 1–11% (overall 1–2%), Togo <1–2%, Nigeria 1–4% (overall 0.8%), Cameroon <1–2%,
East Africa	Very rare	Kenya 19–34%, Tanzania 2%	Rare in Kenya, Uganda and Tanzania, 2% in Mauritius and Reunion Island
Central Africa	Very rare	Central African Republic 39% (23% of pygmy population), Republic of the Congo 36–40% (29% of pygmy population)	Central African Republic <1%, Republic of the Congo <1% (Pygmy population of 6.5%)
Southern Africa	Very rare in South African black populations	Zambia 20–27%, Malawi 39%, Namibia 11.5%, South African Cape Coloured population 7%, South African black 12% (San) to 36% (Venda), Mozambique 5–6%, Madagascar <1–3%, Comoros 2% or more	Comoros 3%,
Africans in UK		Probably 25–30%	0.9%
African-Americans		25%	1–2%
African-Caribbeans	Rare (but recognised in West Indians with Chinese ancestry)	Overall 25%, Jamaica 34%	0.5–10% (overall 1%), Jamaica 1% [72] and 4–5% reported, Lesser Antilles <1–10%, Guadeloupe 0.5%, 0.9% in African-Caribbeans in UK
Mexico			0.4% [73]; up to 15% reported
Afghanistan			3%
Pakistan		15–20%	5%, about 4.5% in UK Pakistanis
Nepal		6–14% (but up to 97% in some tribal populations)	13%

(Continued on pp. 102–103.)

Table 3.5 *Continued.*

Country or ethnic group	α^0 thalassaemia	α^+ thalassaemia	β thalassaemia
India	Rare	5–33% (17–99%, mainly above 50%, in tribal populations)	Overall 1–16% (average around 3%, but up to 40% in some tribal populations), about 3.5% in UK Indians
Bangladesh			3%
Indian subcontinent populations in Britain			Overall, about 4.5% (from 3% in UK Bangladeshis and Punjabi Sikhs to about 6% in East African Asians)
Sri Lanka		15–16% (about 13% $-\alpha^{3.7}$ and about 2% $-\alpha^{4.2}$); 3–20% $-\alpha^{3.7}$ and 0–2.8% $-\alpha^{4.2}$ in different districts [74]	1–5% (overall 2.2%); 0–16.4% in different districts [74]
Maldives			16% [73]
Japan		< 1%	Rare
Korea			Very low
Hong Kong	4.5%	0.5–1%	3%
Singapore	3–4%	8%	
China	3–9% in southern China	< 1%–6% in southern China	0.5% in north and north-west, 2–6% in south, overall c. 1.7%
Chinese in UK			About 3%
Taiwan	3.5% (higher and lower in different aboriginal populations)	2%	1–3%
Myanmar (previously Burma)			0.5–6% (overall 3%); 8% in one study [75]
Thailand	4% in central Thailand, 14% in northern Thailand	3–17%, 3% around Bangkok, 9% in north, 17% in north-east; 1–8% haemoglobin Constant Spring	4–11% (overall 3%)
Cambodia	1–4%	12–28%, haemoglobin Constant Spring up to 2%	1–5% (overall about 2.8%)
Laos	4%	about 14%, Hb Constant Spring 9%	1–9% (overall 5%)
Vietnam	2–3% (southern Vietnam)	About 8%, ~17–22% and haemoglobin Constant Spring 0–4% in southern Vietnam	1–25% (overall 4%)

Table 3.5 *Continued.*

Country or ethnic group	α^0 thalassaemia	α^+ thalassaemia	β thalassaemia
Malaysia	3–9%	Hb Constant Spring <1–6%, overall up to 29%	1–5% (overall 2%)
Philippines	10%	5%	1–2%
Brunei			2%
Indonesia	2.6–3.2%	6% (2–30% on different islands)	0–11% (overall 3%)
Papua New Guinea		10% in highlands, 62% in lowlands	1–25%
Solomon Islands		45%	
Vanuatu (previously New Hebrides)		45%	
New Caledonia		6%	
New Zealand (Maori)		5–10%	
Australia (indigenous)		6%	Rare
Indigenous Americans			Rare
Brazil			Overall 1%

is found in Oceania and the Indian subcontinent. The highest prevalence of the less common but more serious α^0 thalassaemia is found in southern China and South-East Asia.

α^+ thalassaemia heterozygosity, compound heterozygosity and homozygosity

α^+ thalassaemia trait is the most common monogenic disorder in the world. It usually results from deletion of all or part of the $\alpha 2$ globin gene. The most common variants causing α^+ thalassaemia are $-\alpha^{4.2}$ and $-\alpha^{3.7}$. $-\alpha^{4.2}$ is a 4.2 kb deletion including the $\alpha 2$ gene. $-\alpha^{3.7}$ designates a group of three slightly different 3.7 kb deletions of the 3' end of the $\alpha 2$ gene and the 5' end of the $\alpha 1$ gene with formation of an $\alpha 2\alpha 1$ fusion gene. Both $-\alpha^{4.2}$ and $-\alpha^{3.7}$ result in about a 50%

reduction in α chain synthesis from the affected chromosome. Although the $\alpha 2$ gene is usually responsible for about 70% of α chain production there is some upregulation of the $\alpha 1$ gene when the $\alpha 2$ gene is deleted ($-\alpha^{4.2}$) whereas the $\alpha 2\alpha 1$ fusion gene is downregulated in comparison with a normal $\alpha 2$ gene. About a quarter of individuals with African ancestry are heterozygous for α^+ thalassaemia (having the genotype $-\alpha^{3.7}/\alpha\alpha$) while 1–2% are homozygotes (having the genotype $-\alpha^{3.7}/-\alpha^{3.7}$). Other ethnic groups in which deletional α^+ thalassaemia occurs include Greeks, Cypriots, Turks, Sardinians, Lebanese, Saudi Arabs, Indians, Thais, Filipinos, Indonesians, Melanesians and Polynesians. Less often, α^+ thalassaemia is found to result from a small mutation rather than a large deletion of the $\alpha 2$ or $\alpha 1$ gene, referred to as non-deletional α thalassaemia and

designated $\alpha^T\alpha$ or $\alpha\alpha^T$. However, many cases, particularly those affecting the $\alpha 1$ gene, are likely to be unrecognised so that the true frequency is unknown. Non-deletional α thalassaemia affecting the $\alpha 2$ gene leads to a more marked reduction of α globin production than $\alpha 2$ deletion, as there is no upregulation of the $\alpha 1$ gene. Consequently, the haematological abnormality is more marked. $\alpha^T\alpha$ is most common in the Middle East, particularly in Saudi Arabia ($\alpha^{TSaudi}\alpha$) but also in Cyprus and Sardinia. Non-deletional α thalassaemia affecting the $\alpha 1$ gene, $\alpha\alpha^T$, is milder than $\alpha^T\alpha$, usually being intermediate in severity between $-\alpha/\alpha\alpha$ and $--/\alpha\alpha$.

Some α chain variants are also synthesised in reduced amounts and so give rise to the phenotype of α^+ thalassaemia (see Table 3.3). The most common non-deletional α thalassaemia is haemoglobin Constant Spring with a frequency of 1–8% in Thailand. This results from mutation of the STOP codon of the $\alpha 2$ gene so that a further 31 amino acids are added to the α chain; the mRNA is very unstable and the rate of α chain synthesis reduced to about 1% of normal [77]. Haemoglobin Paksé has a similar significance and cannot be distinguished from haemoglobin Constant Spring on high performance liquid chromatography (HPLC), cellulose acetate electrophoresis or capillary electrophoresis.

α^+ thalassaemia is of little significance to the individual since it is clinically silent. Heterozygosity, and to a greater extent homozygosity, for α^+ thalassaemia offer partial protection against severe malaria although the incidence of mild malaria in young children is actually increased [78]. There also appears to be protection against other infections severe enough to cause hospitalisation in children [78]. The condition is of some genetic significance since people who are compound heterozygotes for deletional α^+ thalassaemia and α^0 thalassaemia have haemoglobin H disease (see later). Homozygotes for more severe non-deletional α^+ thalassaemia ($\alpha^T\alpha/\alpha^T\alpha$) can also have the clinical features of haemoglobin H disease. Homozygosity for termination codon mutations, haemoglobin Constant Spring,

haemoglobin Koya Dora and haemoglobin Icaria, leads to more anaemia than is usual in homozygosity for α^+ thalassaemia and a different haematological picture. There is usually a mild haemolytic anaemia and some cases have mild jaundice and hepatosplenomegaly. The haemolysis is the result of membrane damage by oxidised abnormal α chains [27]. Deletion of *LCRA* also leads to a severe phenotype, equivalent to α^0 .

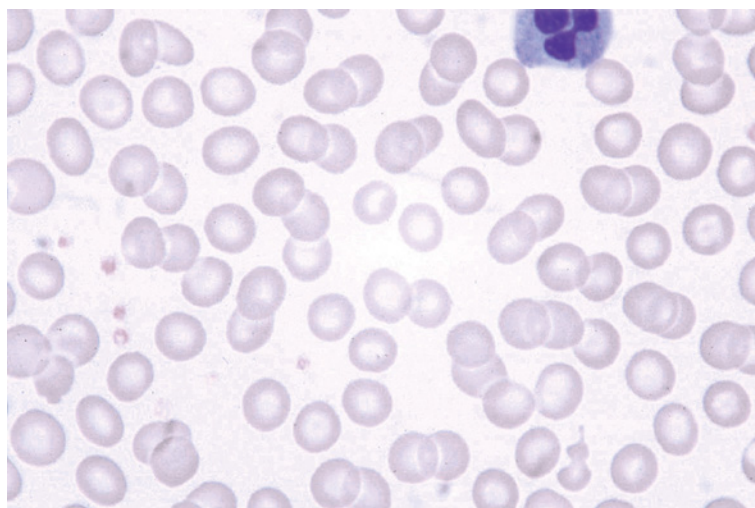
Laboratory features

Heterozygotes for α^+ thalassaemia ($-\alpha/\alpha\alpha$) may have a completely normal blood count and film or trivial anaemia and microcytosis with slight reduction of the mean cell volume (MCV) and mean cell haemoglobin (MCH) and a raised red cell count (RBC). On average, the haemoglobin concentration (Hb) is probably about 10 g/l lower than in subjects with four α genes. All haematological variables – RBC, Hb, haematocrit (Hct), MCV and MCH – show considerable overlap with normal values but mean levels differ. Homozygotes ($-\alpha/-\alpha$) have more marked haematological abnormalities (Figs 3.3 and 3.4), which are usually comparable with those seen in β thalassaemia heterozygotes. In a comparison of healthy adult African-Americans, those with $\alpha\alpha/\alpha\alpha$, $-\alpha/\alpha\alpha$ and $-\alpha/-\alpha$ were found to have mean MCVs of 89.6, 85 and 75.7 fl (men) and 90.3, 84.2 and 72.3 fl (women) respectively [79]. In a large survey of Kenyan children, the presence of sickle cell trait was found to ameliorate the effect of α^+ thalassaemia on the RBC, Hb, MCV and MCH [80]. Haemoglobin electrophoresis or HPLC is normal with the haemoglobin A_2 percentage being normal or reduced.

During pregnancy, haematological changes in women with α thalassaemia trait mirror those in normal women. On average, the Hb falls about 14 g/l, the MCV rises by about 5% and the MCH rises by about 7% [81].

Heterozygotes for haemoglobin Constant Spring have more marked anaemia than is usual in α^+ thalassaemia trait but the MCV is not proportionately reduced [77] and may be normal. Basophilic stippling is usually prominent

Fig. 3.3 Blood film of an adult male α^+ thalassaemia homozygote (genotype $-\alpha^{3.7}/-\alpha^{3.7}$); red cell indices were red cell count (RBC) $5.77 \times 10^{12}/l$, haemoglobin concentration (Hb) 139 g/l, haematocrit (Hct) 0.44, mean cell volume (MCV) 76 fl, mean cell haemoglobin (MCH) 24.1 pg, mean cell haemoglobin concentration (MCHC) 318 g/l. May-Grünwald-Giemsa (MGG) $\times 100$ objective.



(Fig. 3.5) [9, 82] but this is not invariable [83]. Homozygotes for this variant haemoglobin are usually anaemic (Hb around 100 g/l) with a reduction of the MCH (on average around 26 pg) [82]. However the mean MCV is normal or low normal (in one study averaging 88 fl) [82], i.e. considerably less reduced than would be expected, given the degree of reduction of the MCH; it has been postulated that this is because damage to red cell membranes by oxidised α^{CS} globin chains leads to cellular overhydration. The mean cell haemoglobin concentration (MCHC) tends to be low (mean 300 g/l) [82]. Damage to the red cell membrane leads to a considerably shortened red cell survival and reticulocytosis (reticulocytes usually around 6–10%). Splenomegaly is common. There is usually 2–11% haemoglobin Constant Spring and often 1–3% haemoglobin Bart's but no haemoglobin H [9, 82]. The variant haemoglobin is detectable on HPLC and capillary electrophoresis (Fig. 3.6a,b). In occasional cases haemoglobin Constant Spring is lower or even undetectable [84]. Haemoglobin A₂ tends to be low [84]. Haemoglobin F is normal. Homozygotes for haemoglobin Constant Spring generally have mild haemolytic anaemia and splenomegaly. However, fetal anaemia requiring intrauterine transfusion for hydrops fetalis has been described, as has neonatal jaundice necessitating phototherapy [85, 86] with there

being only mild anaemia from several months after birth [85]. Compound heterozygosity for haemoglobin Constant Spring and haemoglobin Paksé can similarly cause hydrops fetalis [86].

In a homozygote for haemoglobin Icaria, there was no increase of the RBC, a normal MCV and a reduced MCH [87].

Increased red cell zinc protoporphyrin, which has been used as a screening test for iron deficiency, has been found in 20% of cases of α thalassaemia trait [88, 89]. Elevation tends to be less than in iron deficiency but there is some overlap so that this test is not reliable in distinguishing between these two conditions. The $-\alpha/\alpha\alpha$ and $-\alpha/-\alpha$ genotypes are associated with increased soluble transferrin receptor [90] indicating increased erythropoiesis.

At birth, neonates with α^+ thalassaemia trait have a lower mean Hb than other neonates. In one study the mean level was 151 g/l in heterozygotes and 141 g/l in homozygotes, in comparison with a mean normal of 154 g/l [91]. The MCV and MCH were similarly reduced. The mean MCV was 100 and 94 fl in comparison with a normal mean of 105 fl. The MCH was 33 and 31 pg in comparison with a mean normal of 35 pg. In one study using HPLC, some but not all babies with heterozygous α^+ thalassaemia had around 1–2% of haemoglobin Bart's, in comparison with 0.5–1.0% in neonates with four

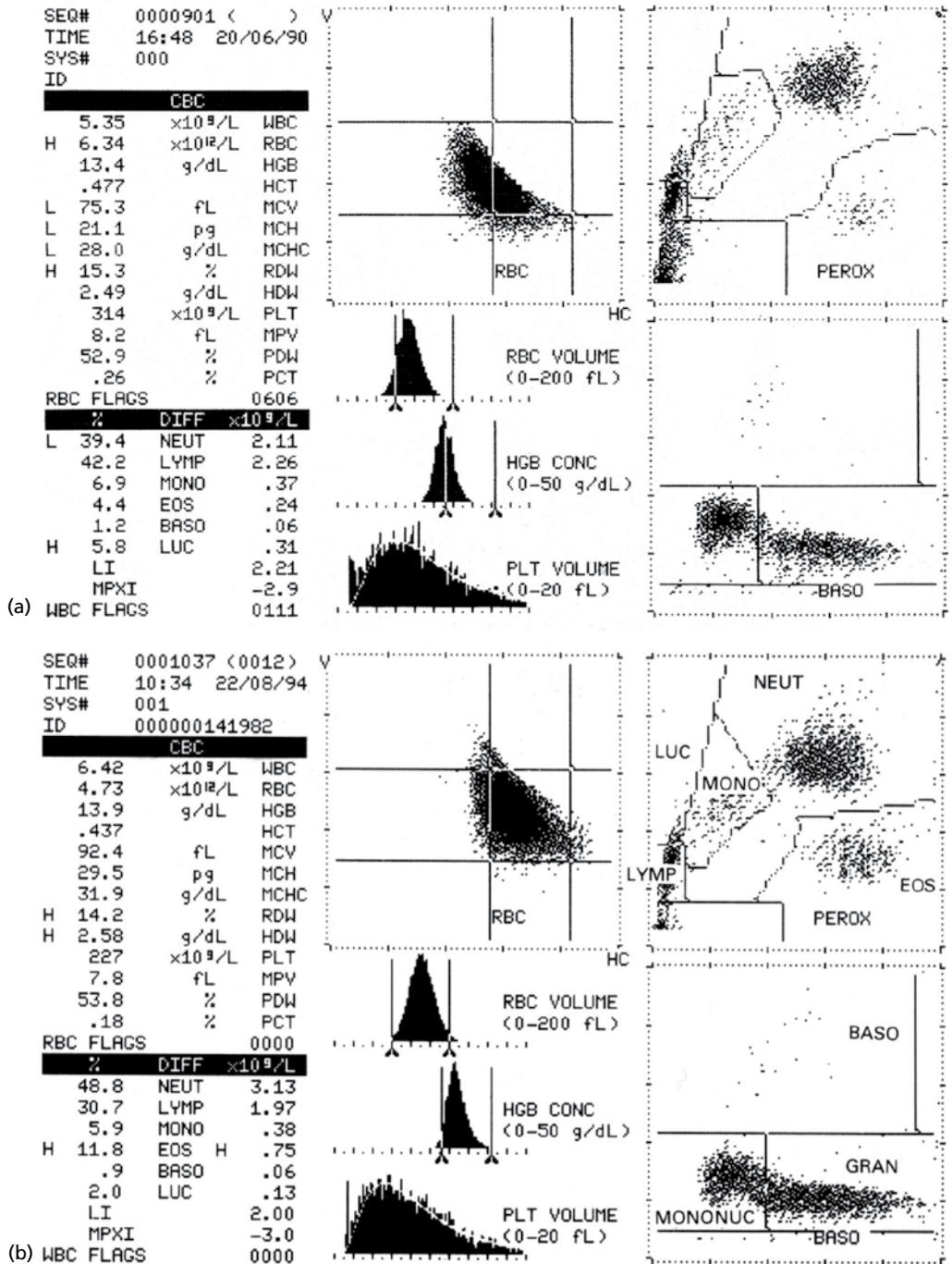


Fig. 3.4 Red cell cytograms and histograms on a Technicon H2 instrument of (a) a male α thalassaemia trait homozygote and (b) a haematologically normal control subject; α thalassaemia is associated with microcytosis that is more marked than the associated hypochromia.

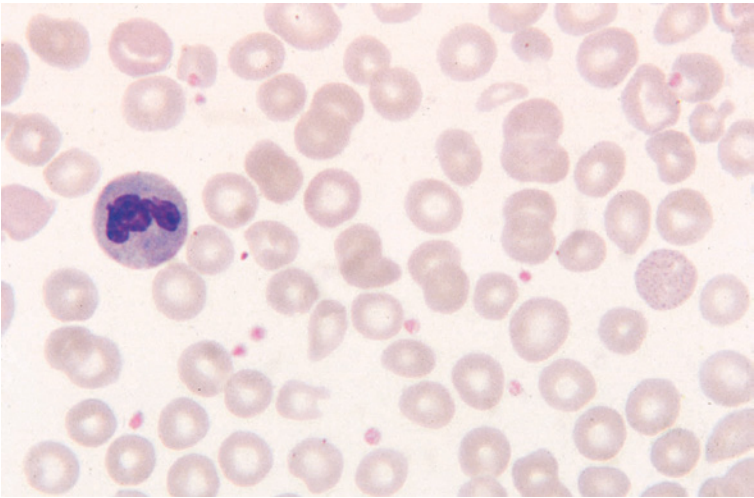


Fig. 3.5 Blood film of a haemoglobin Constant Spring heterozygote showing basophilic stippling. MGG $\times 100$.

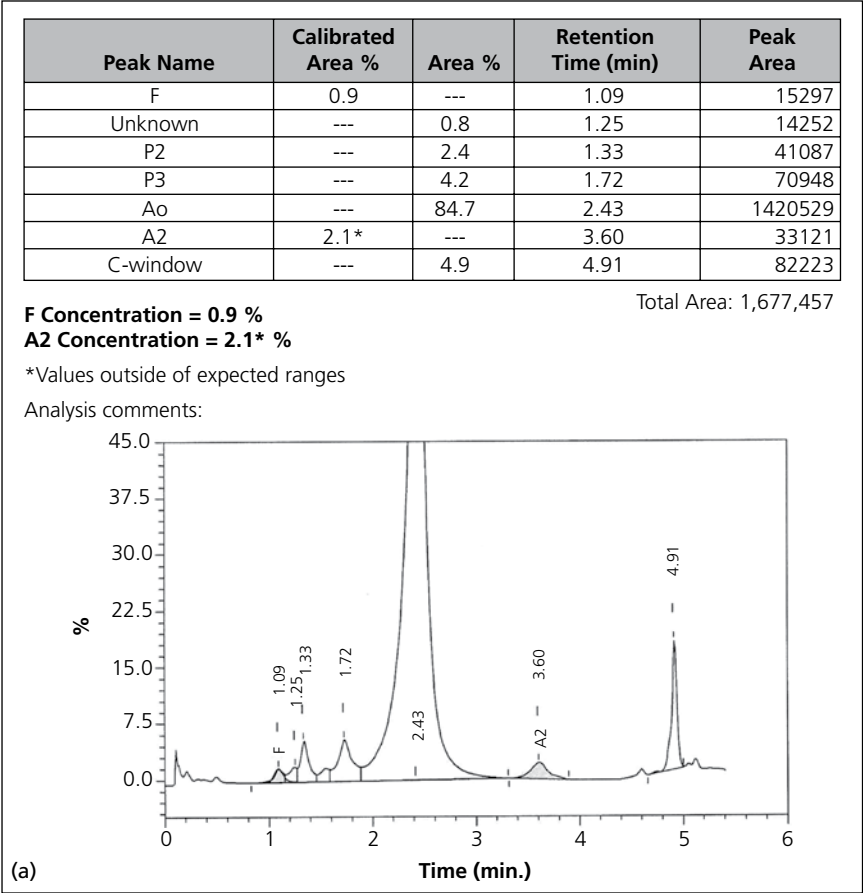


Fig. 3.6 Investigations of a 29-year-old woman who was homozygous for haemoglobin Constant Spring. Red cell indices were RBC $4.37 \times 10^{12}/l$, Hb 111 g/l, MCV 87.2 fl, MCH 25.4 pg and MCHC 292 g/l. (a) High performance liquid chromatography (HPLC) (Bio-Rad variant II) showing three peaks in or near the C window; (Continued on p. 108.)

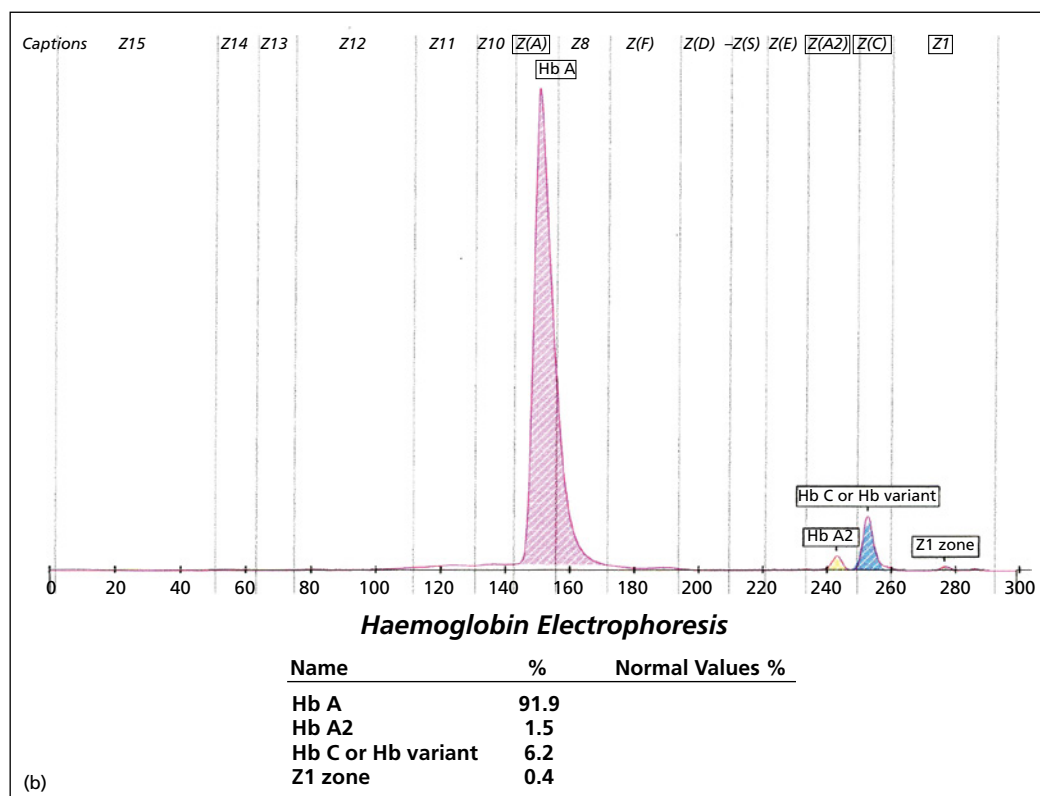


Fig. 3.6 Continued. (b) capillary electrophoresis (Sebia Capillarys) showing a peak in the C zone.

α genes. However, a minority (less than 10%) of babies with four α genes have haemoglobin Bart's up to 1.2–2.6% [91]. In this study there was a difference in haemoglobin Bart's levels between heterozygotes and homozygotes for α^+ thalassaemia – mean values 1.8 and 6.4% respectively – but overlap between heterozygotes and homozygotes occurred [91]; the homozygotes had around 3–10% haemoglobin Bart's. A second HPLC study found 0.8–2.8% haemoglobin Bart's in heterozygotes in comparison with 2.5–8% in homozygotes but again overlap occurred [92]. In a third HPLC study, α^+ thalassaemia heterozygotes had haemoglobin Bart's of 1.55–5% whereas homozygotes had 5–12% with no overlap [93]. The percentage of haemoglobin Bart's is affected by the nature of the α^+ thalassaemia mutation, being higher for $-\alpha^{4.2}$ than for $-\alpha^{3.7}$ [94]. Individuals with α^+ thalassaemia homozygosity have a lower haemoglobin Bart's percentage than those with α^0

thalassaemia heterozygosity, 2.5–8.0% in comparison with 7.6–12.3%, even though both have only two α genes [92]. Haemoglobin Bart's disappears by 3–6 months of age.

Diagnosis

The diagnosis of α thalassaemia trait is usually suspected when an individual is found to have microcytosis that is not explained by β or $\delta\beta$ thalassaemia trait or iron deficiency. (However, it should be noted that there are alternative explanations for 'thalassaemic' red cell indices with normal percentages of haemoglobins A_2 and F; other explanations, which will be discussed later, include coinheritance of β and δ thalassaemia, normal- A_2 - β thalassaemia, $\gamma\delta\beta$ thalassaemia and polycythaemia vera complicated by iron deficiency.) Haemoglobin electrophoresis and HPLC are normal except for a tendency to a reduction of the A_2 percentage.

Very occasional cells may contain haemoglobin H inclusions but this is not a reliable diagnostic test even in those who are homozygous for α^+ thalassaemia. Definitive diagnosis, if needed, requires DNA analysis.

In the neonate, a haemoglobin Bart's concentration of 1–2% on haemoglobin electrophoresis is suggestive of $-\alpha/\alpha\alpha$ but not all neonates with this genotype have elevated haemoglobin Bart's. On HPLC, haemoglobin Bart's must be distinguished from a peak due to bilirubin. With the Bio-Rad Variant II instrument, haemoglobin Bart's has a retention time of about 0.1 minute whereas that of bilirubin, with which it may be confused, is slightly less. If there is any difficulty making the distinction, plasma should be removed from the blood sample before repeating the analysis. Elevation is more likely in association with $-\alpha^{4.2}$ than in association with $-\alpha^{3.7}$. A concentration of >2% of haemoglobin Bart's on haemoglobin electrophoresis of neonatal blood is suggestive of either $--/\alpha\alpha$ or $-\alpha/-\alpha$ and in an ethnic group in which $--/\alpha\alpha$ does not occur provides presumptive evidence of the $-\alpha/-\alpha$ genotype. When the more sensitive technique of HPLC is used, the detection of increased haemoglobin Bart's in a neonate is suggestive of α thalassaemia trait but, because of the detection of haemoglobin Bart's in some normal babies, cannot be regarded as providing a definitive diagnosis.

Since many individuals with α^+ thalassaemia trait have haematological variables falling within the normal range the diagnosis is likely to be unsuspected in many cases unless revealed by population surveys or by family studies of a patient with haemoglobin H disease.

When an α^+ thalassaemia phenotype is consequent on the presence of an α chain variant synthesised at a reduced rate, the variant haemoglobin may be detected by haemoglobin electrophoresis or, more often, HPLC but it comprises a very low proportion of total haemoglobin (e.g. haemoglobin Constant Spring is usually 0.5–1% of total haemoglobin). Haemoglobin Constant Spring can be identified on cellulose acetate electrophoresis at alkaline pH, particularly if a heavy application is used; it moves between carbonic anhydrase and haemoglobin A₂ whereas

haemoglobin A₂' is even slower, moving between the application point and carbonic anhydrase. On HPLC, haemoglobin Constant Spring appears in the C window as one to three very small peaks. In one study at least one abnormal peak was detected in all eight homozygotes and in 38/44 heterozygotes [95]. In the same study, haemoglobin Paksé was not detected by HPLC. In another study haemoglobin Constant Spring appeared as one to three small peaks but sometimes there was no peak apparent and homozygotes could not be distinguished from heterozygotes [96]. Delay in testing beyond three days increases the chance of no peak being apparent [96]. On capillary electrophoresis, haemoglobin Constant Spring and haemoglobin Paksé appear in the C zone. However, an abnormal peak is not always identifiable; in one series of 411 heterozygotes, 4% had no detectable peak and no peak was detectable in 26 patients who were also heterozygous for β thalassaemia [97]. DNA-based techniques can be used for the diagnosis of non-deletional α thalassaemia and are necessary when no variant haemoglobin is detected and diagnosis is important (see Table 2.5).

Coinheritance with other abnormalities of globin chain synthesis

Coinheritance of α^+ thalassaemia, particularly if homozygous, can lessen the laboratory abnormalities of β thalassaemia trait and the clinical and laboratory abnormalities of homozygosity or compound heterozygosity for β thalassaemia. In β thalassaemia trait, the MCV and MCH are higher and the haemoglobin A₂ percentage is lower when α thalassaemia is coinherited. Coexisting α thalassaemia trait reduces the proportion of the variant haemoglobins, including in individuals with sickle cell trait, haemoglobin C trait and haemoglobin E trait.

α^0 thalassaemia trait

α^0 thalassaemia usually results from deletion of both α genes on a single chromosome. Rarely, it results from deletion of only the $\alpha 1$

globin gene and downstream sequences with inactivation of the remaining $\alpha 2$ gene. α^0 thalassaemia is relatively common in Chinese originating in south-eastern China (including Hong Kong), in Taiwan and in South-East Asian populations, specifically in Thailand, Vietnam, Laos, Cambodia, Malaysia, the Philippines, Burma and Indonesia. It occurs at lower frequency in the Mediterranean area – in Greece, Cyprus, Turkey, Israel and certain parts of Italy (e.g. Sardinia). Three types of α^0 thalassaemia trait, designated $--_{SEA}$, $--_{FIL}$ and $--_{THAI}$, are common in South-East Asia (see Fig. 3.2). Two of these, $--_{FIL}$ and $--_{THAI}$, are large deletions that also include the ζ gene. A rare Indian mutation, $--_{KOL}$, also has deletion of the ζ gene [98]. In the Mediterranean area the most common deletion is $--_{MED}$, which does not include the ζ gene, followed in frequency by $-(\alpha)^{20.5}$. The $--_{MED}$ deletion also occurs in the United Arab Emirates, Iran, Yemen, Kuwait and Jordan. In addition, Iran has the $-(\alpha)^{20.5}$ deletion and Yemen has $--_{YEM}$. In the heterozygous state there are no important clinical or haematological differences between the different mutations giving rise to α^0 thalassaemia. The $--_{BRIT}$ mutation is found in Lancashire and also in New Zealand and Newfoundland [99]; the rare deletion designated as $--_{BLACK}$ in African Americans appears to be identical [99].

Laboratory features

α^0 thalassaemia trait leads to very mild anaemia with the Hb overlapping the normal range. The RBC is increased and the MCV and MCH are reduced. The blood film shows microcytosis and a variable degree of hypochromia (Fig. 3.7). The reticulocyte count is elevated to 2–3% [81]. When there is coexisting β thalassaemia trait or triple α in *trans* the MCV and MCH are less abnormal.

At birth, haemoglobin electrophoresis shows neonates with α^0 thalassaemia trait to have 5–10% haemoglobin Bart's. Quantification by HPLC gives values of around 7–11% [91] (Fig. 3.8); the mean value is higher than in homozygosity for α^+ thalassaemia but overlap occurs. Haemoglobin concentration, MCV and MCH are all, on average, lower than in neonates with four α genes or with heterozygosity or homozygosity for α^+ thalassaemia. In one study mean values were 133 g/l, 86 fl and 29 pg, respectively [91].

Diagnosis

α^0 thalassaemia should be suspected when red cell indices suggestive of thalassaemia trait are found in a patient of appropriate ethnic origin with normal percentages of haemoglobins A_2 and F. In this context an MCH of less than 25 pg

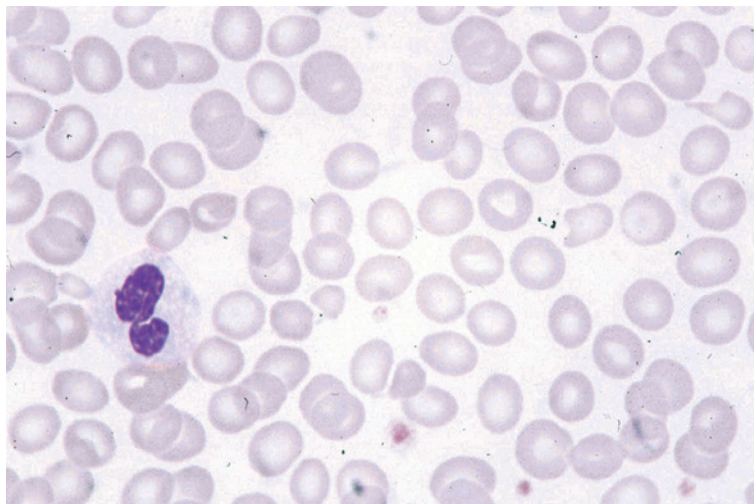


Fig. 3.7 Blood film of a female α^0 thalassaemia heterozygote (genotype $--_{SEA}/\alpha\alpha$); the red cell indices were RBC $5.71 \times 10^{12}/l$, Hb 115 g/l, Hct 0.38, MCV 66 fl, MCH 20.1 pg, MCHC 304 g/l. MGG $\times 100$.

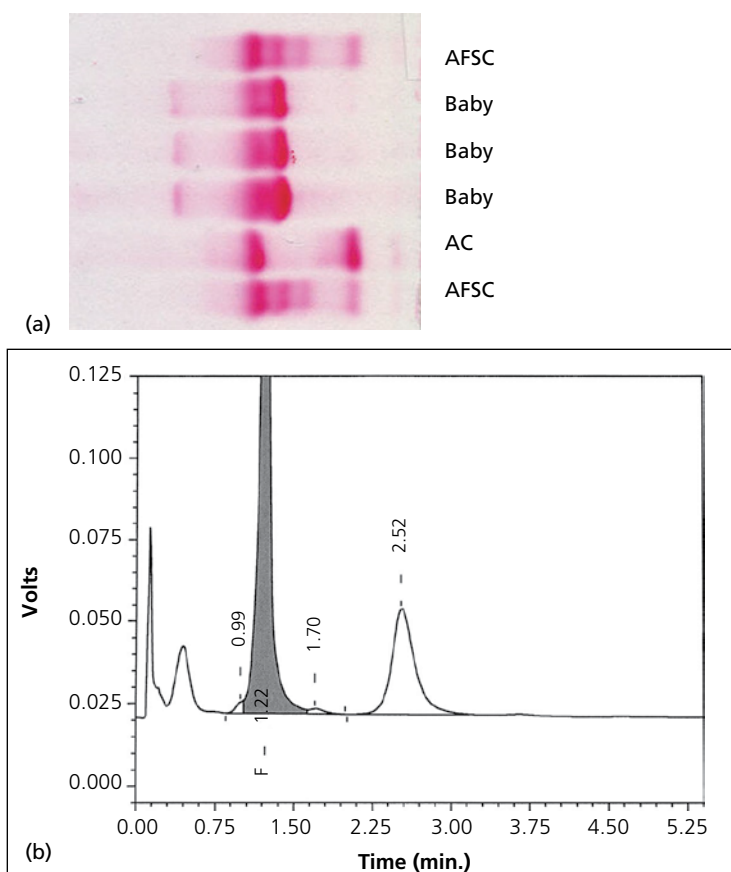


Fig. 3.8 (a) Haemoglobin electrophoresis on cellulose acetate at alkaline pH showing a fast band that is haemoglobin Bart's ('Baby'); AFSC is a control sample containing haemoglobins A, F, S and C; (b) HPLC chromatogram (Bio-Rad Variant II) showing haemoglobin Bart's, superimposed on acetylated haemoglobin F, in a baby of Filipino ancestry with α^0 thalassaemia trait ($-\alpha^{FIL}/\alpha\alpha$); HPLC analysis was done on washed red cells so there is no bilirubin present. From left to right, the peaks are haemoglobin Bart's (tall peak) plus acetylated haemoglobin F (complex peaks), haemoglobin F (dark grey) and haemoglobin A_0 .

suggests a diagnosis of either α^0 thalassaemia trait or homozygosity for α^+ thalassaemia trait rather than α^+ thalassaemia heterozygosity. Definitive diagnosis requires DNA analysis. Because tests for the diagnosis of α thalassaemia are not readily available in many hospitals, it is usual to exclude other causes of a low MCH, such as iron deficiency, β thalassaemia and $\delta\beta$ thalassaemia trait, and the presence of variant haemoglobins before proceeding to DNA analysis. Iron deficiency can be excluded by measuring the serum ferritin concentration but it should be noted that serum transferrin receptor concentration is elevated in α thalassaemia

trait [90] as well as in iron deficiency so is not a useful test in this context. Zinc protoporphyrin may be elevated; values tend to be lower than in iron deficiency although there is some overlap [89]. The detection of rare haemoglobin H inclusions in red cells can also be useful in the diagnosis of α^0 thalassaemia trait (Fig. 3.9). However this test is time consuming and both false negative and false positive results can occur. The number of cells with haemoglobin H inclusions is reduced by concomitant β thalassaemia trait or haemoglobin E trait. It is therefore reasonable, in low prevalence areas, not to test for haemoglobin H inclusions but to

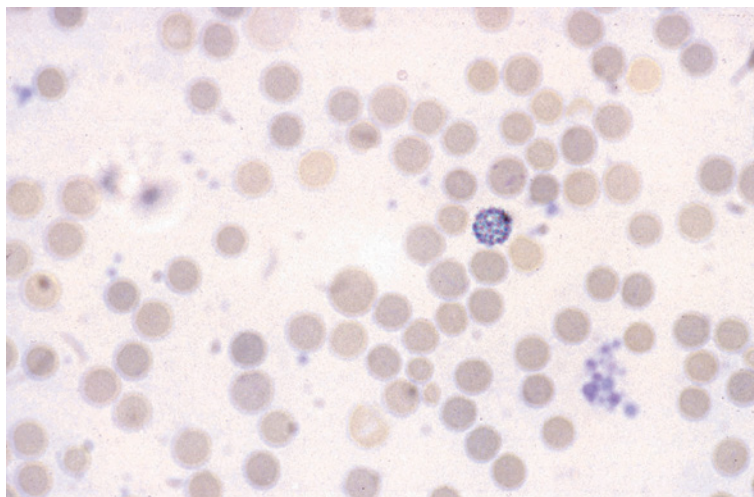


Fig. 3.9 Haemoglobin H preparation from a patient with α^0 thalassaemia trait showing a single cell containing haemoglobin H inclusions. Brilliant cresyl blue $\times 40$.

proceed straight to DNA analysis if accurate diagnosis is important (e.g. in a pregnant woman and her partner with suspected α^0 thalassaemia trait). In high prevalence areas, particularly if resources are limited, the test has been found useful. However, an immunochromatographic strip for the detection of haemoglobin Bart's (i+MED Laboratories) can be used for screening [100–102] and has been found to be more sensitive and specific than screening for haemoglobin H inclusions. Some false negatives have been reported [100, 101] but in one study all 19 cases of heterozygous α^0 thalassaemia were detected [102].

In the neonatal period, a significantly elevated percentage of haemoglobin Bart's (5–10%) on haemoglobin electrophoreses or HPLC is compatible with a diagnosis of α^0 thalassaemia trait but is not diagnostic since similar levels are seen in homozygosity for α^+ thalassaemia trait. When quantitated by HPLC, the mean percentage is higher than in α^+ thalassaemia heterozygosity (9.2% in comparison with 6.4%) but again overlap occurs [91]. Haemoglobin Bart's disappears by 3–6 months of age. α^0 thalassaemia caused by the $--^{SEA}$ mutation can be detected by demonstrating an increased percentage of ζ chains, e.g. by slot blot immunobinding or enzyme-linked immunosorbent assay (ELISA). The latter may be practicable for screening purposes in areas where the majority of α^0 thalassaemia is caused by this

mutation [103]. Reagents are commercially available [104]. This technique is only of use in countries with a significant incidence of this genotype and DNA analysis will still be necessary if both partners appear likely to have α^0 thalassaemia trait, on the basis of red cell indices, but only one has detectable ζ chain.

Coinheritance with other abnormalities of globin chain synthesis

Inheritance of α^0 thalassaemia trait has a similar effect to homozygosity for α^+ thalassaemia on the phenotype of coinherited β thalassaemia, sickle cell trait, haemoglobin C trait and haemoglobin E trait (see earlier). In ethnic groups in which haemoglobin E and α^0 thalassaemia are both common, it is necessary to consider the possibility of their coexistence, particularly when screening pregnant women. One study indicated that if the haemoglobin E plus A_2 (as measured by HPLC) was less than 21.5%, testing for α^0 thalassaemia was indicated [105]. We would advise testing if haemoglobin E plus A_2 is less than 25%. Coinheritance with α^+ thalassaemia or with a hyperunstable α chain variant usually leads to typical haemoglobin H disease (see below) but sometimes to a more severe, transfusion-dependent condition [106].

In relation to antenatal screening of pregnant women, it should also be noted that the

presence of β thalassaemia trait does not exclude the simultaneous presence of α^0 thalassaemia trait. Failure to detect both abnormalities may lead to a failure to predict haemoglobin Bart's hydrops fetalis when one partner has α^0 thalassaemia trait and the other has both α^0 and β thalassaemia trait. In ethnic groups in which α^0 thalassaemia occurs, DNA analysis of both partners is indicated if one partner has β thalassaemia trait and the other has probable α^0 thalassaemia trait [107]. The increase in the MCV and MCH if α^0 thalassaemia trait and triple α are coinherited ($- - / \alpha\alpha\alpha$) may mean that occasional cases of α^0 heterozygosity are missed [108].

Haemoglobin H disease

Haemoglobin H disease is a clinical syndrome resulting from a variety of genetic abnormalities leading to one functional α globin gene or its equivalent (Table 3.6) [14, 20–24, 29, 109–114]. The most common cause is compound heterozygosity for α^+ thalassaemia and α^0 thalassaemia (e.g. $- -^{SEA} / - \alpha^{4.2}$ in South East Asia and $- (\alpha)^{20.5} / - \alpha^{3.7}$ or $- -^{MED} / - \alpha^{3.7}$ in the Mediterranean). Alternative mechanisms are (i) compound heterozygosity for α^0 thalassaemia and a non-deletional α thalassaemia such as haemoglobin Constant Spring, haemoglobin Paksé or the Saudi type of non-deletional α thalassaemia

Table 3.6 Examples of genetic abnormalities leading to haemoglobin H disease [derived from references [14, 20–24, 29, 109–114] and other sources]; this list is not exhaustive.

$- - / - \alpha$	Compound heterozygosity for α^0 and α^+ thalassaemia, e.g. $- -^{SEA} / - \alpha^{4.2}$, $- -^{SEA} / - \alpha^{3.7}$ in China and South-East Asia, $- (\alpha)^{20.5} / - \alpha^{3.7}$, $- -^{MED} / - \alpha^{3.7}$ in the Mediterranean area and $- -^{SA} / - \alpha^{3.7}$ or $- -^{SA} / - \alpha^{4.2}$ in India
$- - / \alpha^T \alpha$	Compound heterozygosity for α^0 thalassaemia and non-deletional α thalassaemia, e.g. $- -^{MED} / \alpha^{-Snt} \alpha$ and $- -^{MED} / \alpha^{TSaudi} \alpha$ in the Mediterranean area
$- - / \alpha \alpha^T$	Compound heterozygosity for α^0 thalassaemia and non-deletional α thalassaemia, e.g. $- - / \alpha \alpha^{VSI-117G^A}$ [111]
$\alpha^T \alpha / \alpha^T \alpha$	Homozygosity for non-deletional α thalassaemia. e.g. $\alpha^{TSaudi} \alpha / \alpha^{TSaudi} \alpha$ or $\alpha^{NCO} \alpha / \alpha^{NCO} \alpha$ in the Mediterranean area
$\alpha^T \alpha^T \alpha / \alpha^T \alpha$	Rare example of haemoglobin H disease with a total of 5 α genes, 3 of which carry the α^{TSaudi} mutation
$\alpha^{TSaudi} \alpha / \alpha^{TSaudi} \alpha$, $\alpha^{PA2} \alpha / \alpha^{PA2} \alpha$ and $\alpha^{TSaudi} \alpha / - \alpha^{3.7}$	Homozygosity for non-deletional α thalassaemia affecting the dominant $\alpha 2$ gene* or compound heterozygosity for non-deletional α thalassaemia and deletional α thalassaemia trait in the Middle East
$- - / \alpha^{CS} \alpha$, $\alpha^{CS} \alpha / \alpha^{CS} \alpha$ or $- - / \alpha^{Paksé} \alpha$	Compound heterozygosity for α^0 thalassaemia and either haemoglobin Constant Spring or haemoglobin Paksé, e.g. $- -^{SEA} / \alpha^{CS} \alpha$ or $- -^{SEA} / \alpha^{Paksé} \alpha$ in South-East Asia† or homozygosity for $\alpha^{CS} \alpha$ in South-East Asia or the Middle East
$- - / \alpha^{QS} \alpha$	Compound heterozygosity for α^0 thalassaemia and haemoglobin Quong Sze (non-deletional α thalassaemia), e.g. $- -^{SEA} / \alpha^{QS} \alpha$ in South-East Asia
Genotype not certain	Compound heterozygosity for α^0 thalassaemia and an unstable haemoglobin, haemoglobin Petah Tikva‡
$\alpha^{TSaudi} \alpha / \alpha^{Agrinio} \alpha$	Compound heterozygosity for non-deletional α thalassaemia and haemoglobin Agrinio, a haemoglobin with an unstable or rapidly catabolised α chain, in Greeks and Greek Cypriots; haemoglobin H percentage is relatively low but disease may be severe [112]
$- - / \alpha^{Agrinio} \alpha$	Compound heterozygosity for α^0 thalassaemia and haemoglobin Agrinio giving severe disease in occasional Cypriots [112]
$- \alpha^{3.7} (-AC) /$ $- \alpha^{3.7} (-AC)$	Homozygosity for ACCATG \rightarrow $- -$ CATG mutation superimposed on $- \alpha^{3.7}$ in North Africa and Mediterranean area

(Continued on p. 114.)

Table 3.6 Continued.

--/- α	Compound heterozygosity for α^0 and α^+ thalassaemia, e.g. -- _{SEA} /- $\alpha^{4.2}$, -- _{SEA} /- $\alpha^{3.7}$ in China and South-East Asia, - (α) ^{20.5} /- $\alpha^{3.7}$, -- _{MED} /- $\alpha^{3.7}$ in the Mediterranean area and -- _{SA} /- $\alpha^{3.7}$ or -- _{SA} /- $\alpha^{4.2}$ in India
--/- α^Q	Compound heterozygosity for α^0 thalassaemia and α^Q on a chromosome with a - $\alpha^{4.2}$ deletion, haemoglobin H/Q disease
--/ $\alpha^{G\text{Philadelphia}}$ -	Compound heterozygosity for α^0 thalassaemia and $\alpha^{\text{Philadelphia}}$ occurring on a chromosome on which the other α gene is deleted; only haemoglobins G, G ₂ and H are present, haemoglobin H/G Philadelphia disease
--/ $\alpha\alpha^{21\text{nt insertion}}$	Compound heterozygosity for α^0 thalassaemia and a 21 base pair insertion in the $\alpha 1$ gene causing a variant haemoglobin and transfusion-dependent disease [113]
-- _{Poly A1} /- $\alpha\alpha^{21\text{nt insertion}}$	Compound heterozygosity for an $\alpha 1$ polyadenylation mutation and a 21 base pair insertion in the $\alpha 1$ gene causing severe haemoglobin H disease [113]
($\alpha\alpha$)/- α	Compound heterozygosity for deletion of the upstream regulatory region and α^+ thalassaemia
($\alpha\alpha$) ^{ALT} /-- _{SEA}	Compound heterozygosity for α^0 thalassaemia and deletion of the upstream regulatory region, R2 [114]
($\alpha\alpha$) ^T /($\alpha\alpha$) ^T	Mutation creating a new GATA1 binding site between the locus control region and the α genes that out-competes the α gene promoters for the locus control region [29], Melanesian haemoglobin H disease
($\alpha\alpha$) ^{Jx} /- α	Compound heterozygosity for deletion of enhancer of γ and α genes (<i>LCRA</i> , MCS-R2) and α^+ thalassaemia
($\alpha\alpha$) ^{Jx} /-- _{SEA}	Compound heterozygosity for deletion of enhancer of γ and α genes (HS -40, MCS-R2) and α^0 thalassaemia (very severe haemoglobin H disease) [24]
- $\alpha^{3.7}$ /- α^{Souli}	Compound heterozygosity for α^+ thalassaemia and haemoglobin Souli (unstable rapidly degraded α chains) [20]
$\alpha^{\text{Dartmouth}}$ α /- $\alpha^{\text{Dartmouth}}$ α and $\alpha^{\text{Dartmouth}}$ α /-- _{SEA}	Both transfusion dependent [21]
Homozygosity for 2 nucleotide deletion in polyadenylation signal	Sometimes transfusion dependent [22] but not usually [23]
Mutation of the <i>ATRX</i> gene at Xq13.3 encoding a <i>trans</i> -acting factor	<i>ATRX</i> syndrome of mild haemoglobin H disease, mental retardation and facial and genital dysmorphism
Acquired somatic mutation of the <i>ATRX</i> gene	Acquired haemoglobin H disease in haematological neoplasms

* α^{TSaudi} is also known as α^{PA1} .

† Other termination codon mutations can also interact with α^0 thalassaemia to cause haemoglobin H disease, e.g. haemoglobin Icaria [8].

‡ It is not known whether the mutation is in the $\alpha 2$ or $\alpha 1$ gene; other very unstable α chains or haemoglobins can also interact with α^0 thalassaemia to produce haemoglobin H disease, e.g. haemoglobins Suan-Dok [14], Evanston [14], Adana [14], Pak Num Po [109] and Zurich-Albisrieden [110]. It appears that compound heterozygosity for two unstable α chain variants, Zurich-Albisrieden and Sallanches, can likewise lead to the phenotype of haemoglobin H disease [110].

(e.g. $-\text{SEA}/\alpha^{\text{CS}}\alpha$, $-\text{SEA}/\alpha^{\text{Paksé}}\alpha$ or $-\text{MED}/\alpha^{\text{TSaudi}}\alpha$) – often referred to as ‘non-deletional haemoglobin H disease’; (ii) compound heterozygosity for α^0 thalassaemia and a highly unstable α chain such as $-\text{SEA}/\alpha^{\text{QS}}\alpha$ (haemoglobin Quong Sze); and (iii) homozygosity or compound heterozygosity for non-deletional α thalassaemia, mainly $\alpha^{\text{TSaudi}}\alpha/\alpha^{\text{TSaudi}}\alpha$ or $\alpha^{\text{TSaudi}}\alpha/\alpha^{\text{Agr}}\alpha$. Rare non-deletional cases are caused by mutation of a gene on the X chromosome, leading to the ATRX syndrome. Whether $-\text{SEA}/\alpha^{\text{WS}}\alpha$ when haemoglobin Westmead is coinherited with α^0 thalassaemia should be classified as haemoglobin H disease is uncertain since the condition is milder; only half the patients are anaemic, some have a normal MCV and MCH and a haemoglobin H band is not detected on electrophoresis [115].

Haemoglobin H disease is characterised by a greatly reduced rate of synthesis of α chain, leading to a hypochromic microcytic haemolytic anaemia. The excess of β chain production over α chain production leads to formation of an abnormal haemoglobin with β chain tetramers, referred to as haemoglobin H. Haemoglobin H functions poorly from the point of view of oxygen delivery to tissues; it lacks haem–haem interaction, so that the oxygen dissociation curve is hyperbolic rather than sigmoid, and it has a very high oxygen affinity. Haemoglobin H is soluble but it is prone to oxidation and then becomes unstable, precipitating to some extent in erythroblasts with resultant intramedullary cell death (ineffective erythropoiesis) [109]. Oxidised haemoglobin H precipitates in circulating erythrocytes, becoming attached to and damaging the red cell membrane with resultant membrane rigidity, red cell fragmentation and chronic haemolytic anaemia [77, 109]. Erythrophagocytosis by splenic macrophages is attributable in part to membrane rigidity and in part to increased binding of immunoglobulin to damaged membranes so that the cells are then recognised by the Fc receptors of macrophages. Ineffective erythropoiesis occurs but is less marked than in β thalassaemia major because unpaired β globin chains cause less damage to erythroblasts than free α chains. Haemolysis is thus more important than ineffective

erythropoiesis as a cause of anaemia. In one study of eight patients mean red cell survival was reduced to about a quarter of normal (22.5 days in comparison with a mean of 97 days in five normal controls) [116].

In haemoglobin H disease due to deletion of three genes, the disease tends to be more severe when *HBA2* is deleted than when *HBA1* is deleted. In general, disease is also more severe when there is a non-deletional α thalassaemia or a highly unstable α chain (e.g. $-\text{SEA}/\alpha^{\text{TSaudi}}\alpha$ or $-\text{SEA}/\alpha^{\text{QS}}\alpha$ respectively) than when there is simple gene deletion, $-\text{SEA}/-\alpha$. In one study of more of 338 patients the mean Hb was 75 g/l in comparison with 90 g/l [115]. In another study of 37 patients, non-deletional haemoglobin H disease (mainly $-\text{SEA}/\alpha^{\text{CS}}\alpha$) and $-\text{SEA}/\alpha^{\text{QS}}\alpha$ had a similar Hb to deletional disease (mainly $-\text{MED}/-\alpha^{3.7}$ or $-\text{MED}/-\alpha^{3.7}$) but the effective haemoglobin concentration was lower because of a higher percentage of haemoglobin H (mean 29% cf. 6.9%); there was also more splenomegaly [117]. Splenectomy is more likely to be carried out in non-deletional cases [118]. In another study, children with haemoglobin H/Constant Spring disease ($-\text{SEA}/\alpha^{\text{CS}}\alpha$) had slower growth and, although the MCV and MCH were higher, the Hb was lower, the reticulocyte count and plasma bilirubin were higher and there was a much greater risk of severe anaemia requiring blood transfusion [119]. In a study in Thailand, in the presence of a non-deletional mutation, genotype mainly $-\text{SEA}/\alpha^{\text{CS}}\alpha$, there was more anaemia, splenomegaly and growth failure [120]. In a series of patients in Iran, none of the 31 patients with deletional haemoglobin H disease had required transfusion whereas of 18 patients with non-deletional haemoglobin H disease, there were six who were regularly transfused, four who were occasionally transfused, two who had been transfused once only and only six who had not been transfused [121]. In the case of $-\text{SEA}/\alpha^{\text{CS}}\alpha$ there is also a greater degree of ineffective erythropoiesis as a result of damage to developing erythroblasts by precipitated Constant Spring α chains [122]. The serum ferritin is significantly higher in non-deletional cases [115, 117]. However, the ratio of serum ferritin to hepatic iron is lower in haemoglobin H/Constant Spring than in deletional haemoglobin H disease, which

needs to be taken into account if serum ferritin is used to indicate the need for chelation therapy [122].

Even greater severity is seen if there is a highly unstable α chain produced, as with $--/\alpha^{QS}\alpha$ [109], $--/\alpha^{SD}\alpha$ or $--/\alpha\alpha^{\text{Pak Num Po}}$, so that there is not only precipitation of haemoglobin H but also damage to developing erythroblasts by precipitated unstable α chain.

The presence of a gain-of-function mutation in the *PIP4K2A* gene has been found to increase β globin synthesis, leading to a lower Hb and more numerous haemoglobin H inclusions than expected for the α gene phenotype [123].

Haemoglobin H disease may present clinically with splenomegaly and symptoms of anaemia. Reduced height, body weight and body mass index have also been observed [124]. It should be noted that, because of the high oxygen affinity of haemoglobin H, symptoms will be more severe than would otherwise be expected from the haemoglobin concentration. The anaemia is aggravated by infection, pregnancy or exposure to oxidant drugs. Sudden worsening of anaemia can be due to increased haemolysis for which the bone marrow cannot compensate. Increases in the rate of haemolysis can be caused by infection, inflammation or drugs. Acute intravascular haemolysis can occur [125]. Rarely acute haemolysis induced by infection causes very severe anaemia, shock and renal failure. A sudden worsening of anaemia can also be due to superimposed folate deficiency or transient erythroblastopenia following parvovirus B19 and other viral infections. Some patients are jaundiced and the incidence of gallstones and cholecystitis is increased. In one study, 11 of 72 patients had gallstones [115] and in another study, 12 of 23 [126]. Some patients have leg ulcers. There is an increased incidence of osteopenia and osteoporosis. The outcome of pregnancy may be affected adversely; in one study there was an increased incidence of intrauterine growth restriction, preterm birth and low birth weight [127].

Often the clinical features of haemoglobin H disease are so mild that the diagnosis is made incidentally. In other patients the anaemia is much more severe and intermittent or, rarely,

regular transfusions are required (three of 251 patients in one series) [128]. Transfusion may become necessary only later in life. In severely affected patients, there is growth retardation and expansion of the bone marrow cavity leading to bony deformity affecting the facial bones, similar to that which is seen in β thalassaemia major. Liver iron overload is frequent, being observed in a third of patients (median age 53 years) in one study, with 20% having advanced fibrosis and 9% probable cirrhosis; these complications were more frequent with non-deletional α thalassaemia [129]. Other consequences of iron overload include diabetes mellitus, cardiomyopathy and endocrine dysfunction [109]. In another study four of 338 patients had a ferritin of greater than 1000 ng/ml and required iron chelation therapy [115]. Some patients undergo splenectomy to relieve hypersplenism or because of recurrent episodes of severe anaemia [119]. There is a considerably increased risk of post-splenectomy thrombosis [130]. The incidence of pulmonary hypertension is increased [131]. Very rarely, the phenotype of haemoglobin H disease is even more severe, with hydrops fetalis leading, in some cases, to death *in utero* or soon after birth [132]. Those babies who survive the perinatal period may have disease of moderate severity, but with transfusion independence in later life, or may remain transfusion dependent. This severe phenotype of haemoglobin H disease usually results from compound heterozygosity for α^0 and non-deletional α thalassaemia affecting the $\alpha 2$ gene (e.g. $--^{MED}/\alpha^{TSaudi}\alpha$) [132, 133] but can also result from compound heterozygosity for α^0 thalassaemia and a severe α^+ thalassaemia phenotype resulting from a very unstable α chain such as haemoglobin Quong Sze ($--^{SEA}/\alpha^{QS}\alpha$) [134] or haemoglobin Adana [135] ($--^{FIL}/\alpha^{Adana}\alpha$ or $-(\alpha)^{20.5}/\alpha^{Adana}\alpha$) or from homozygosity for non-deletional α thalassaemia (e.g. $\alpha^{Tindian}\alpha/\alpha^{Tindian}\alpha$) [136], $\alpha^{CS}\alpha/\alpha^{CS}\alpha$ [137], $\alpha^{Agr}\alpha/\alpha^{Agr}\alpha$ [138] or $\alpha^{Adana}\alpha/\alpha^{Adana}\alpha$ [59]. Fetal hydrops has also been reported with $--^{SEA}/\alpha^{CS}\alpha$ [139]. These genotypes do not necessarily lead to hydrops fetalis, although this may be the usual outcome with $\alpha^{Adana}\alpha/\alpha^{Adana}\alpha$ [59]. The phenotypic variation with a single genotype is not well understood.

Haemoglobin H disease with haemoglobin Constant Spring is more severe than deletional haemoglobin H disease. In one large study, pregnancy was associated with more preterm birth, fetal growth restriction and lower birth weight in both groups, with the haemoglobin H-Constant Spring group pregnancies being more severely affected [140].

In summary, haemoglobin H disease is most severe if there is homozygosity or compound heterozygosity for non-deletional α thalassaemia, slightly less severe if there is compound heterozygosity for deletional and non-deletional thalassaemia and least severe if there is deletion of three of the four α genes [141, 142]. Most of the non-deletional α chain mutations described in haemoglobin H disease have been in the $\alpha 2$ gene but occasional examples are in the $\alpha 1$ gene [141, 142]. In one study the functional defect in non-deletional haemoglobin H disease was found to be similar whether the mutation was in the $\alpha 1$ or $\alpha 2$ gene; it was suggested that this was because transcription of an abnormal gene interfered with transcription of the normal gene [141]. In another series, both patients with a non-deletional defect of the $\alpha 1$ gene had mild disease whereas those with a non-deletional defect of the $\alpha 2$ gene were generally severe [142].

In the case of the ATRX syndrome, the haemoglobin H disease is relatively mild and the clinical presentation is with dysmorphism and intellectual disability in young boys. Diagnosis usually relies on demonstrating the presence of haemoglobin H inclusion bodies. There is short stature, microcephaly, facial dysmorphism and abnormalities of the external genitalia. Most patients with this syndrome have been Caucasian but five Japanese cases have also been reported [143–145].

Patients with haemoglobin H disease may respond to therapy with mitapivat, a stimulator of pyruvate kinase activity, with a rise in Hb and reduced bilirubin and lactate dehydrogenase (LDH) [146].

Laboratory features

There is a moderately severe hypochromic microcytic anaemia with the Hb varying from

30 to 110 g/l (usually 70–100 g/l) (Fig. 3.10). The MCV is of the order of 50–65 fl and the MCH usually 15–20 pg. The MCHC is reduced, usually to between 250 and 300 g/l. The reduction in the MCHC reflects not only reduced haemoglobin synthesis but also cellular overhydration resulting from membrane damage; this is particularly so in patients with haemoglobin Constant Spring [77, 109]. When one of the genetic abnormalities leading to haemoglobin H disease is a non-deletional α thalassaemia (e.g. α^{CS} , $\alpha^{Paksé}$ or α^{QS}), the anaemia, reticulocytosis and hypochromia are more marked, the MCHC is lower and the MCV and MCH are significantly higher [77, 109, 117, 147]; the MCV can be near normal as a result of overhydration of cells [27]. In one study the mean MCV was 68.8 fl in comparison with 58.3 fl in deletional cases [115]. In another comparison, cases of non-deletional haemoglobin H disease had a mean MCV of 76 fl (cf. 64 fl in deletional), a mean MCH of 22.1 pg (cf. 20.0 pg) and a mean MCHC of 292 (cf. 313 fl) [117]. The RBC is increased. In patients with hypersplenism there may be a reduction in the white cell count (WBC) and platelet count.

The blood film (Fig. 3.11) shows striking anisocytosis, poikilocytosis, hypochromia and microcytosis. Poikilocytes may include target cells, fragments and tear drop poikilocytes. Basophilic stippling may be present. Nucleated red blood cells can be present but often they are not. The percentage and absolute reticulocyte count are increased. Serum soluble transferrin receptor and erythropoietin concentration are increased. Following splenectomy, preformed haemoglobin H inclusions that are no longer pitted by the spleen may be apparent in erythrocytes [148] (see Fig. 3.21).

The bone marrow aspirate (Fig. 3.12) shows erythroid hyperplasia and micronormoblastic maturation. Ultrastructural examination shows abnormal cytoplasmic inclusions that are likely to represent precipitated β chain (Fig. 3.13).

Serum bilirubin concentration and urinary urobilinogen are increased. The bilirubin is unconjugated. Serum LDH may also be increased. Serum haptoglobin is reduced.

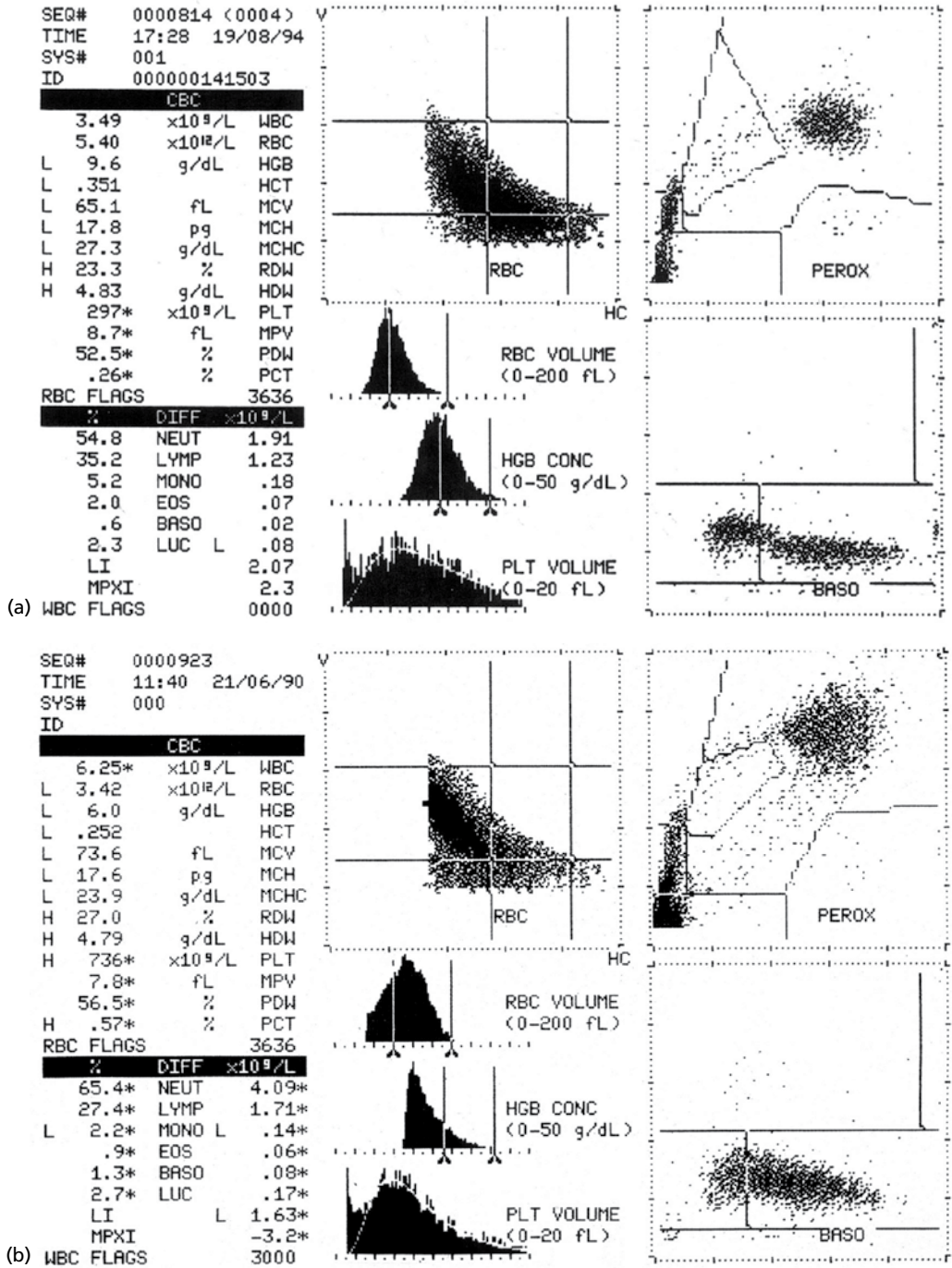


Fig. 3.10 Red cell cytograms and histograms on a Technicon H2 instrument from two patients with haemoglobin H disease: (a) a patient with disease of average severity showing moderately severe anaemia and marked microcytosis and hypochromia; (b) a patient with disease severe enough to have required splenectomy showing marked microcytosis and an extreme degree of hypochromia.

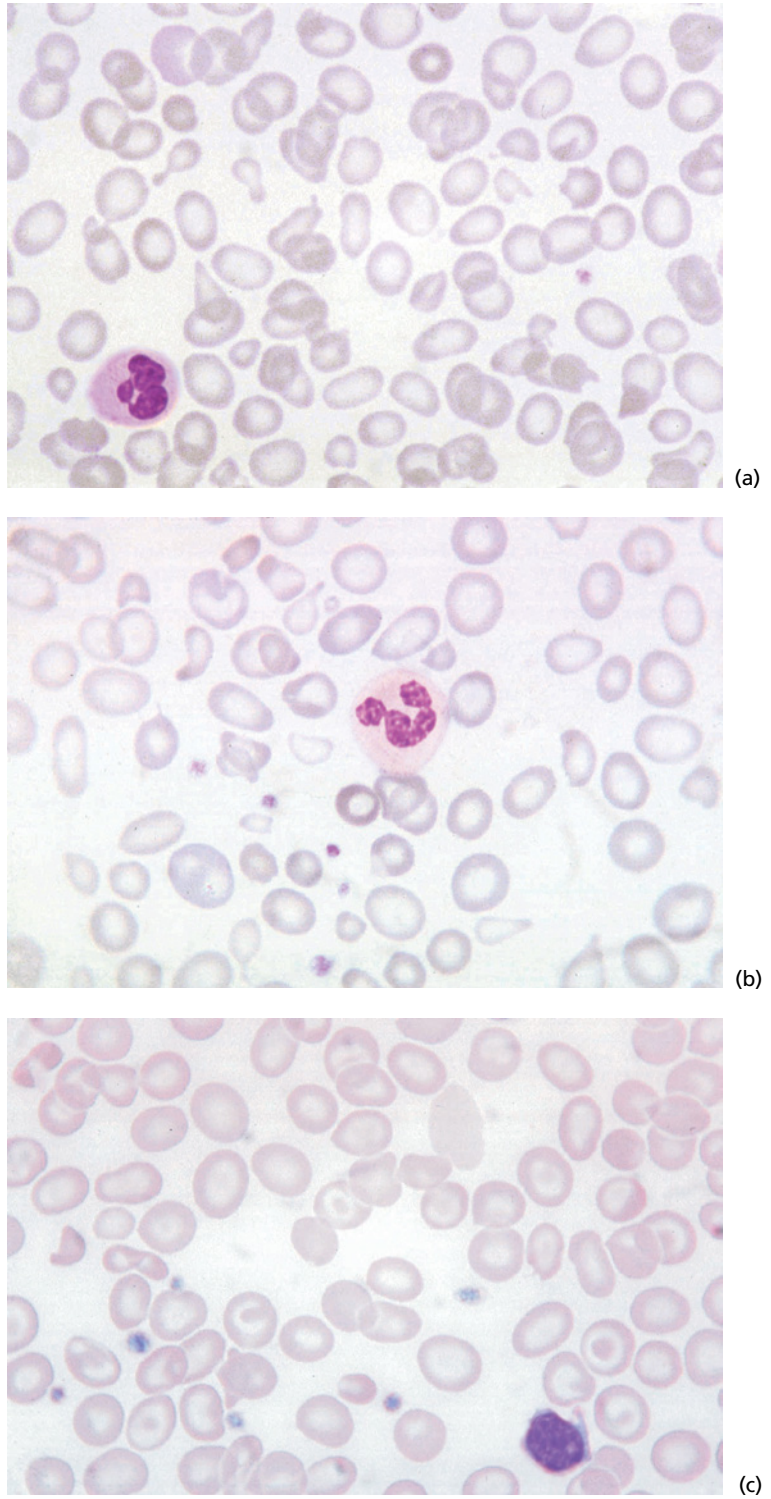
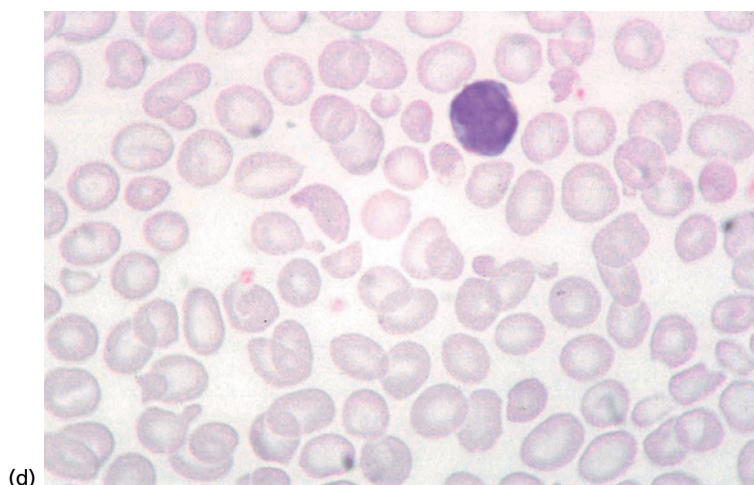


Fig. 3.11 Blood films from four patients with haemoglobin H disease showing the range of abnormality that is observed; (a) red cell indices were RBC $4.95 \times 10^{12}/l$, Hb 96 g/l, Hct 0.30, MCV 60.5 fl, MCH 19.4 pg, MCHC 321 g/l, reticulocyte count $237 \times 10^9/l$; (b) red cell indices and haemoglobin H percentage were RBC $5.34 \times 10^{12}/l$, Hb 93 g/l, Hct 0.34, MCV 63 fl, MCH 17.5 pg, MCHC 277 g/l and haemoglobin H 5%; (c) red cell indices were RBC $5.65 \times 10^{12}/l$, Hb 92 g/l, Hct 0.33, MCV 58.2 fl, MCH 16.4 pg, MCHC 281 g/l; (Continued on p. 120.)



(d)

Fig. 3.11 *Continued.*
 (d) red cell indices were RBC
 $5.34 \times 10^{12}/l$, Hb 104 g/l, Hct
 0.34, MCV 63.6 fl, MCH
 19.5 pg, MCHC 306 g/l.
 MGG $\times 100$.

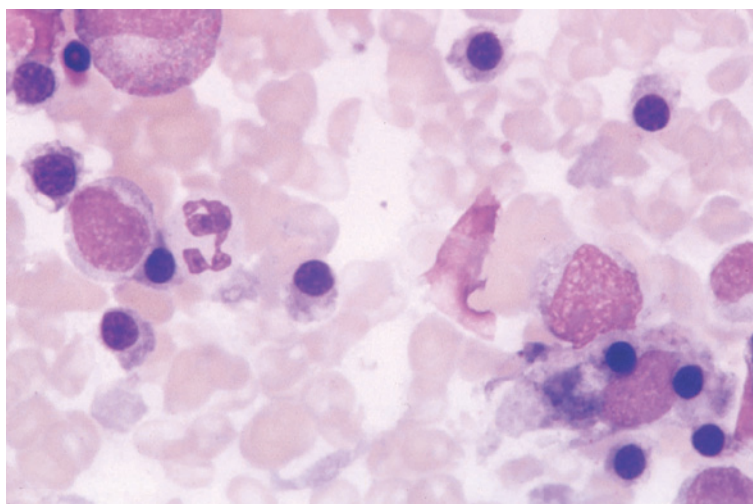


Fig. 3.12 Bone marrow
 aspirate in haemoglobin H
 disease; there is erythroid
 hyperplasia and some
 erythroblasts show defective
 haemoglobinisation.
 MGG $\times 100$.

Assays of glucose-6-phosphate dehydrogenase (G6PD) give higher levels in subjects with haemoglobin H disease, whether expressed per g of haemoglobin or per number of red cells [149]; this may be the result of reticulocytosis. Shortened red cell survival and reticulocytosis are also likely to lead to a lower percentage of glycated haemoglobin (haemoglobin A_{1c}).

Haemoglobin electrophoresis (Fig. 3.14), HPLC (Fig. 3.15), capillary electrophoresis (Fig. 3.16) and isoelectric focusing (IEF) show that haemoglobin H comprises 1–40% of total haemoglobin (usually 8–10%). On HPLC there is a double peak and on capillary electrophoresis there is a single peak. The haemoglobin H percentage is usually higher when one of the

genetic defects is a non-deletional thalassaemia (e.g. a mean of 15% in comparison with a mean of 7% [150], a mean of 29% in comparison with 6.9% [117] and a mean of 12.2% in comparison with 7.1% [147] in three studies) and is lower when there is coexisting heterozygosity for β^S , β^C or β^E [109]. Rarely, no haemoglobin H is apparent [115]. Conversely, the haemoglobin H can be as high as 50% [117]. On HPLC using the Bio-Rad Variant II system, haemoglobin H may be missed if a haemoglobin A_2/A_{1c} dual programme is used, as a result of haemoglobin H appearing early in the haemoglobin F window [151]. Laboratories using the Beta Thal Short programme on this instrument need to inspect the chromatogram carefully



Fig. 3.13 Ultrastructural examination of bone marrow erythroblast from a patient with haemoglobin H disease showing an electron-dense stellate inclusion, probably consisting of precipitated β chain. (By courtesy of the late Professor Sunitha N. Wickramasinghe.)

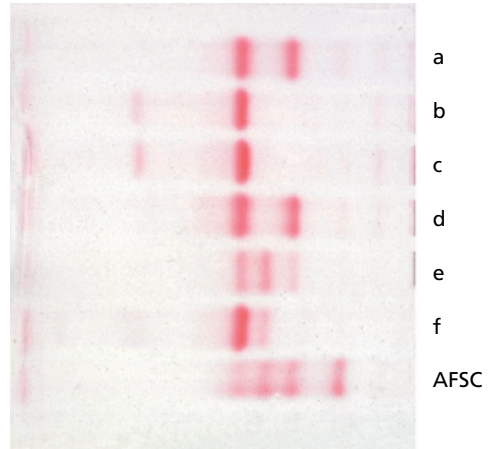


Fig. 3.14 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in a patient with haemoglobin H disease showing a fast band to the left of haemoglobin A (b and c); AFSC is a control sample containing haemoglobins A, F, S and C.

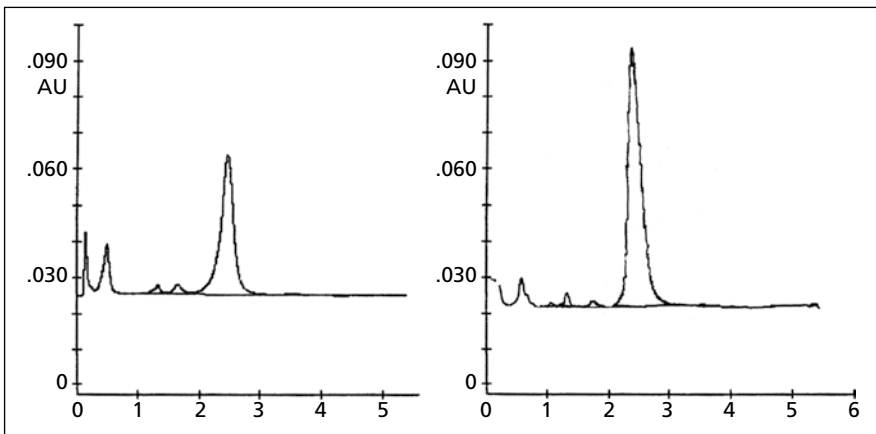


Fig. 3.15 HPLC chromatogram (Bio-Rad Variant) in two patients with haemoglobin H disease; the double peak to the left is haemoglobin H while the major peak on the right is haemoglobin A.

since haemoglobin H is not integrated. Haemoglobin Bart's is present in some patients, usually comprising around 5% of total haemoglobin; at birth it may be 20–40% [109]. It is higher in non-deletional cases, with a mean of 1.61% in comparison with 0.89% [147]. Rarely, when the remaining α gene is mutated no haemoglobin A is present (Fig. 3.17a,b). A very low percentage of haemoglobin H can also be present in the neonate. The percentage of haemo-

globin Bart's in the neonate is higher than the percentage of haemoglobin H later in life, probably because of instability of haemoglobin H (Fig. 3.18a,b). In neonatal screening, 10% haemoglobin Bart's has been used as a threshold to investigate for haemoglobin H disease [152]. The percentage of haemoglobin A_2 is usually reduced (e.g. to 1–2%). It is lower in non-deletional cases (0.67% in comparison with 0.97%) [147]. Haemoglobin F may be

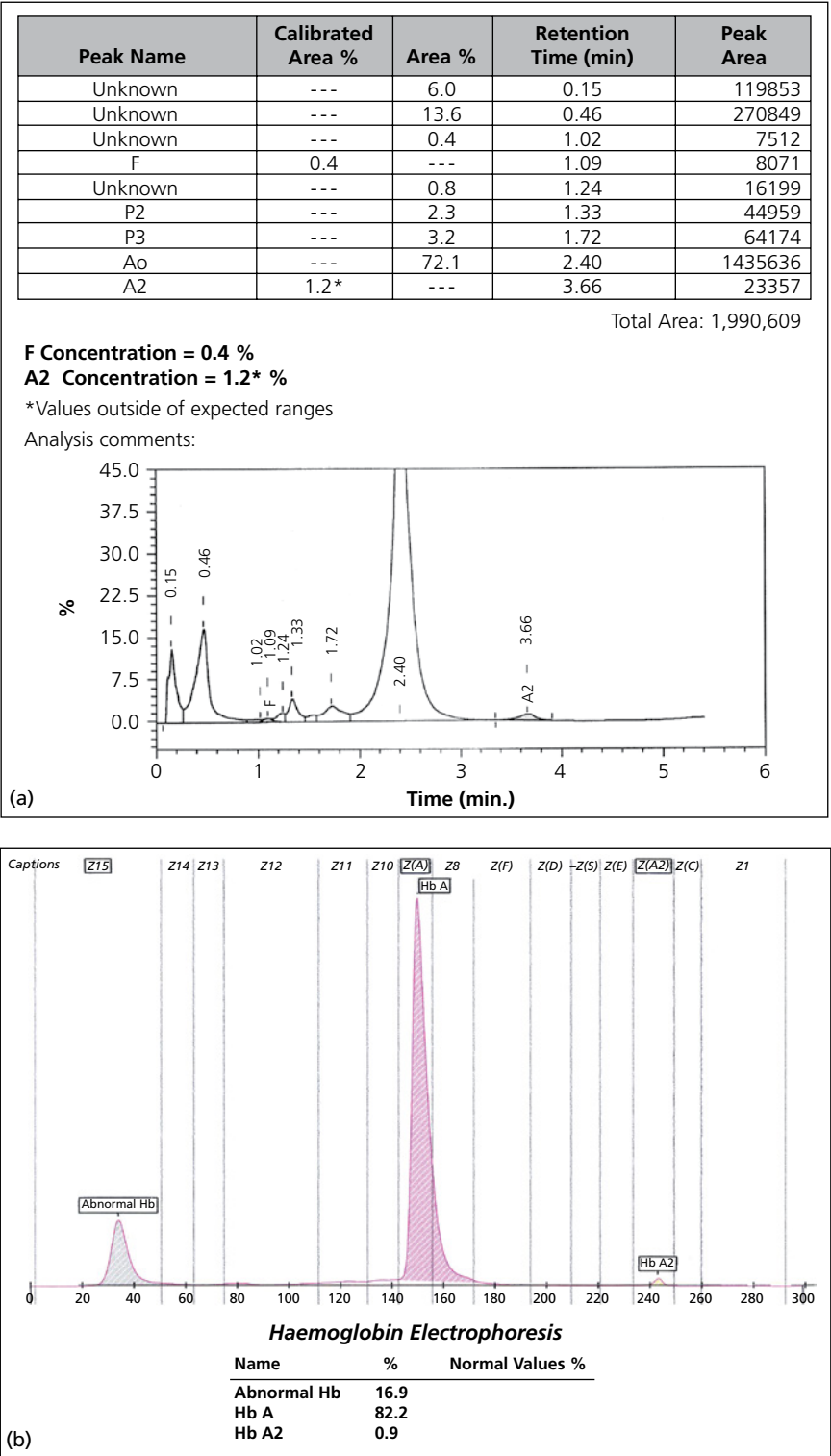


Fig. 3.16 Haemoglobin H disease due to $\alpha^{-3.7}$ plus a large deletion removing the entire α globin gene cluster: (a) HPLC chromatogram (Bio-Rad Variant II) and (b) capillary electrophoresis where the ‘abnormal Hb’ is haemoglobin H (Capillaries, Sebia). Note the low percentage of haemoglobin A₂ that is characteristic of haemoglobin H disease.

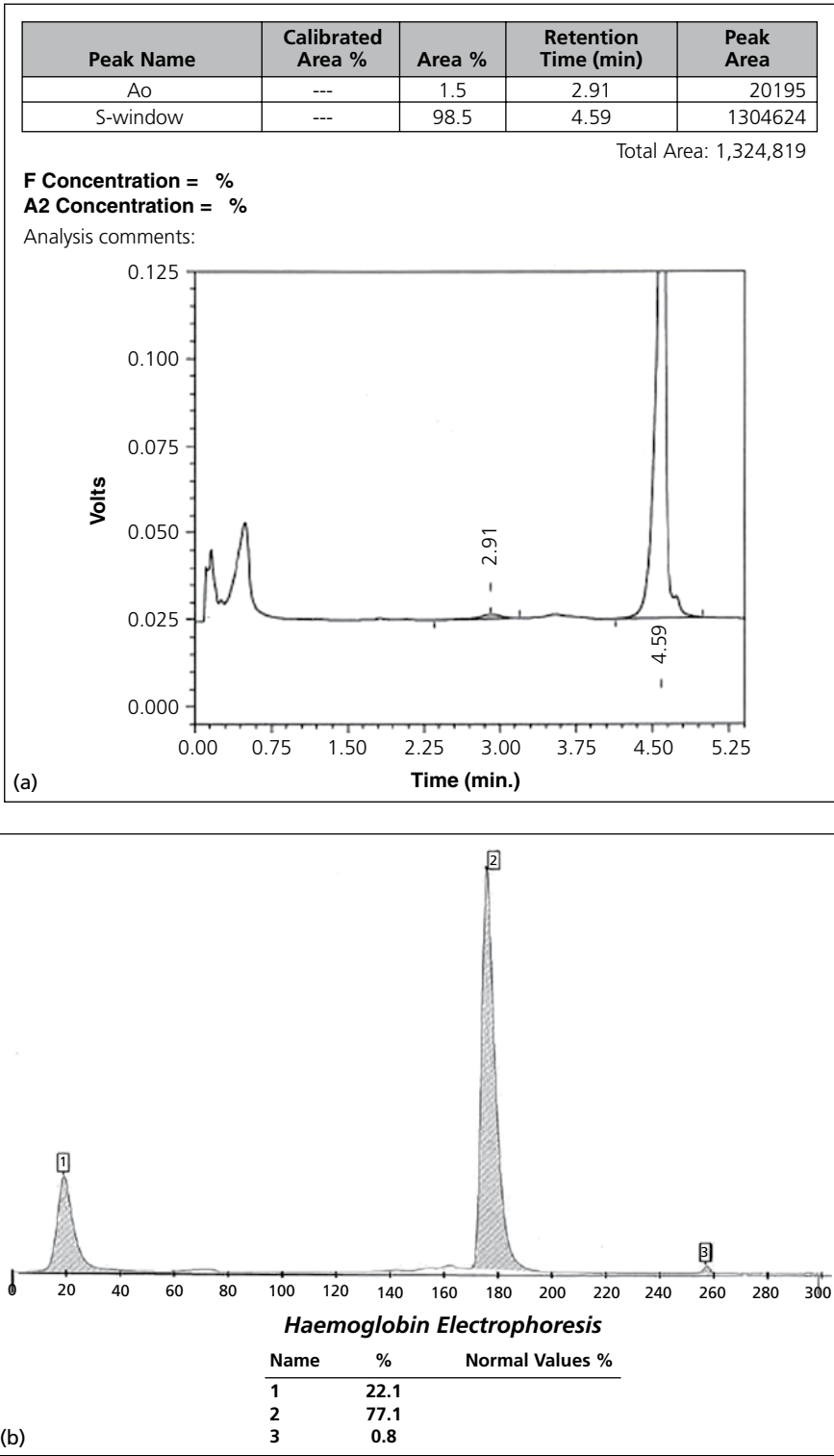


Fig. 3.17 Investigations of a patient with haemoglobin H disease and haemoglobin Q^{Thailand}; (a) HPLC (Bio-Rad Variant II), showing from left to right the double peak of haemoglobin H, which is not integrated, and a Q-Thailand peak; (b) capillary electrophoresis (Sebia Capillarys) showing 22.1% haemoglobin H and 77.1% haemoglobin Q^{Thailand}.

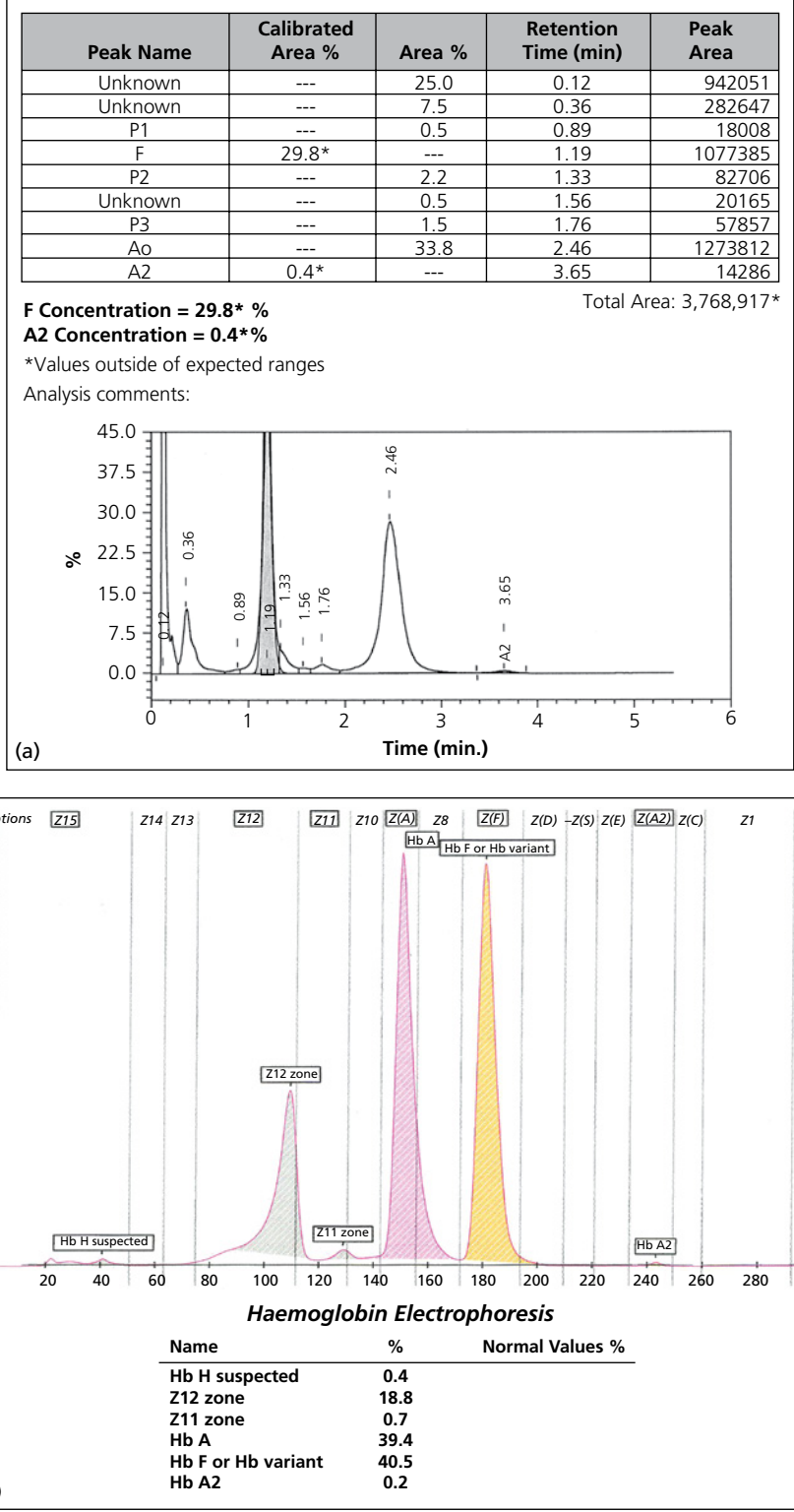


Fig. 3.18 Investigation of a neonate with haemoglobin H disease showing a high percentage of haemoglobin Bart's: (a) HPLC (Bio-Rad variant II), the major peaks from left to right being haemoglobin Bart's (25%), altered haemoglobin F, haemoglobin F and haemoglobin A. Note the very low haemoglobin A₂; (b) capillary electrophoresis (Sebia Capillarys) showing haemoglobin Bart's (18.8%) in the Z12 zone. The genotype was $-\alpha / -(\alpha)^{20.5}$. The red cell indices were RBC $6.11 \times 10^9 / l$, Hb 135 g/l, MCV 76.9 fl, MCH 22 pg and MCHC 287 g/l.

increased to 1–3%. When either haemoglobin Constant Spring or haemoglobin Paksé is present the variant haemoglobin is a very low percentage and haemoglobin Paksé is not detected in all patients in whom it is predicted from molecular data [150]. A haemoglobin H preparation, in which red cells are exposed to a mildly oxidant dye such as brilliant cresyl blue or new methylene blue, shows characteristic ‘golf ball’ haemoglobin H inclusions in a large proportion, usually 35–90%, of red cells (Fig. 3.19). These inclusions, which form *in vitro* during incubation with vital dyes, are shown on ultrastructural examination to be attached to the red cell membrane (Fig. 3.20). In patients who have

been splenectomised there are, in addition, larger preformed Heinz bodies, also attached to the red cell membrane. Sometimes these are apparent in a routine blood film (Fig. 3.21) [148] as well as in a haemoglobin H preparation (Fig. 3.22). Both the percentage of haemoglobin H and the proportion of cells containing haemoglobin H inclusions are lowered by concomitant iron deficiency; haemoglobin H formation may even be completely suppressed [153]. A similar temporary suppression of haemoglobin H synthesis has been reported in one patient with anaemia of chronic disease and in another with alcohol-induced sideroblastic anaemia [154]. When haemoglobin H disease is

Fig. 3.19 Haemoglobin H preparation in a patient with haemoglobin H disease showing both ‘golf ball’ haemoglobin H inclusions and also an increased reticulocyte count. Brilliant cresyl blue $\times 100$.

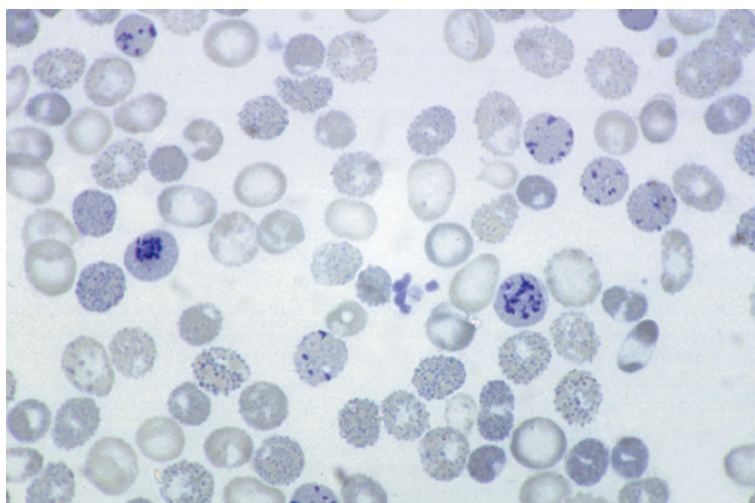
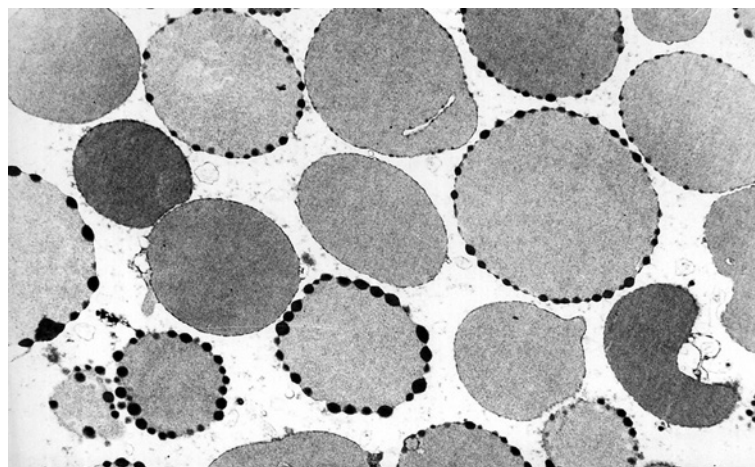


Fig. 3.20 Ultrastructural examination of erythrocytes from a patient with haemoglobin H disease following incubation with brilliant cresyl blue; haemoglobin H inclusions are apparent, attached to the red cell membrane. (By courtesy of the late Professor Sunitha N. Wickramasinghe.)



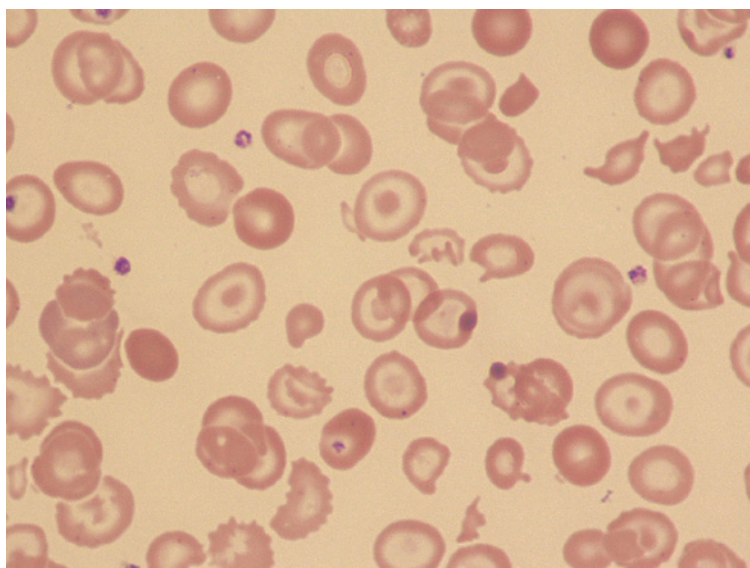


Fig. 3.21 Blood film from a patient with haemoglobin H disease who has had a splenectomy showing a hypochromia, poikilocytosis and a number of preformed Heinz bodies (solid inclusions attached to the red cell membrane). MGG $\times 100$.

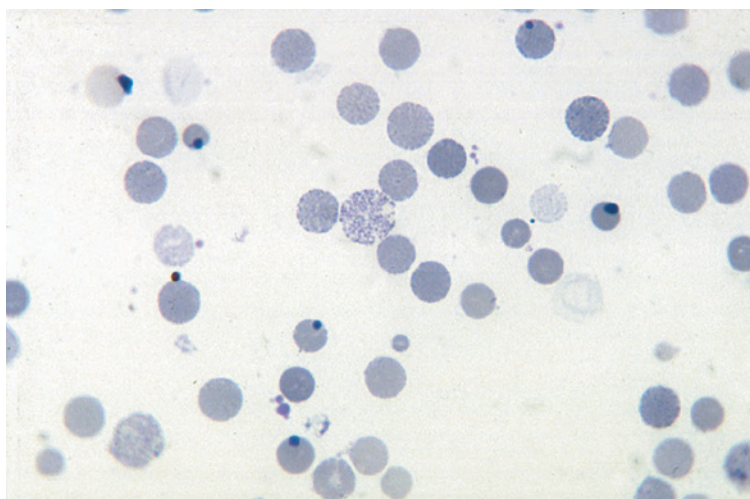


Fig. 3.22 Haemoglobin H preparation from a patient with haemoglobin H disease who has been splenectomised showing 'golf ball' haemoglobin H inclusions, preformed Heinz bodies and an increased reticulocyte count. Brilliant cresyl blue $\times 40$.

caused by non-deletional α thalassaemia, haemoglobin H is not always detectable on haemoglobin electrophoresis and haemoglobin H inclusions may be infrequent; it has been postulated that this may be because β chains and an abnormal α chain are forming transitory and unstable dimers that are rapidly destroyed [13].

Globin chain synthesis studies show an α :non- α ratio of the order of 0.26–0.60 [142]. The oxygen dissociation curve is abnormal. Since haemoglobin H comprises a relatively low percentage of total haemoglobin, the P_{50} may be

normal or only slightly reduced; however, the lower part of the dissociation curve is displaced to the left, giving a biphasic curve.

Haemoglobin H disease occurring as part of the ATRX syndrome is relatively mild. Not all patients are anaemic and the MCH and MCV are sometimes normal. The percentage of haemoglobin H is lower than is usual in haemoglobin H disease and in some cases none is detected on electrophoresis. Haemoglobin H inclusions are detectable in the great majority of cases but in a relatively low proportion of cells (e.g. 0.5–15%).

In occasional cases of haemoglobin H disease, the single remaining α gene encodes a structurally abnormal α chain so that no haemoglobin A is synthesised. There is haemoglobin H, a significant proportion of an α chain variant (e.g. haemoglobin G Philadelphia or haemoglobin Q) and small amounts of a haemoglobin A_2 variant (e.g. G-Philadelphia₂ or Q₂). Less rare is haemoglobin H disease caused by compound heterozygosity for α^0 thalassaemia and non-deletional α^+ thalassaemia consequent on an α chain variant that is synthesised at a greatly reduced rate (e.g. haemoglobin Constant Spring, haemoglobin Paksé or haemoglobin Icaria). In this case small amounts of the variant (e.g. 1.5–2% of haemoglobin Constant Spring) will be present in addition to haemoglobin A and haemoglobin H. Haemoglobin H disease with haemoglobin Paksé is very similar to haemoglobin H disease with haemoglobin Constant Spring and in the past has been misdiagnosed [150]; both have a minor slowly migrating band on electrophoresis and inconspicuous peaks on HPLC. A multiplex allele-specific polymerase chain reaction (PCR) permits the distinction to be made. Genes encoding very unstable α chains can also lead to haemoglobin H disease, either when there is homozygosity or when they are co-inherited with other α thalassaemia variants [10]. Examples include haemoglobin Quong Sze and haemoglobin Suan-Dok.

Neonates with haemoglobin H disease have a lower Hb than other neonates, usually of the order of 120–140 g/l, and a markedly lower MCV and MCH (e.g. 73 and 74 fl and 22 pg in two reported cases [155]). Haemoglobin Bart's comprises 5–40% (usually 20–25%) of total haemoglobin. Haemoglobin H is present as a low percentage. Neonatal screening for haemoglobin H disease can be carried out, as is done in the state of California, by further investigation of babies found to have more than 25% of haemoglobin Bart's at birth [156].

Coinheritance with other abnormalities of globin chain synthesis

Coexisting β thalassaemia trait often makes haemoglobin H disease milder than would

otherwise be the case but some instances of transfusion dependency have been reported [157]. The Hb and MCHC tend to be higher although the MCV and MCH are lower [158, 159]; in one study mean Hb was 113 g/l in comparison with 97 g/l [159]. Haemolysis is less. The reticulocyte count and bilirubin concentration may be normal [157] or the reticulocyte count may be mildly elevated [160]. Serum soluble transferrin receptor is elevated, indicating increased (but ineffective) erythropoiesis [160]. Significant iron overload can occur [157]. The percentage of haemoglobin F and the proportion of cells containing haemoglobin H inclusions are reduced in comparison with those of uncomplicated haemoglobin H disease [155] and sometimes no haemoglobin H can be detected [156]. In one study no haemoglobin H was detected on HPLC in six of 14 cases [158] and in another none was detected on capillary electrophoresis in 28 cases [159]. A trace of haemoglobin Bart's is sometimes detected. The haemoglobin A_2 percentage is sometimes normal and sometimes elevated, but to a lesser extent than is usual in β thalassaemia trait. The α :non- α chain synthesis ratios are of the order of 0.5–0.7, similar to those seen in α thalassaemia heterozygosity [160].

Coinheritance of the genotype of haemoglobin H disease with compound heterozygosity for β^+ and β^0 thalassaemia leads to more balanced chain synthesis so that although there is a moderate anaemia and marked microcytosis, the patient has a clinically mild to moderate condition [161]. Haemoglobin H is not detected but there may be traces of haemoglobin Bart's; haemoglobin A_2 in one patient was 15% [161]. Haemoglobin Bart's in the absence of haemoglobin H has also been reported when there is coinheritance with β^0 thalassaemia heterozygosity [162].

Atypical phenotypes occur when the genotype of haemoglobin H disease is coinherited with haemoglobin S trait (see page 221), haemoglobin C trait (see page 302), heterozygosity for deletional hereditary persistence of fetal haemoglobin (HPFH) (see page 180), non-deletional HPFH (see page 184)

and haemoglobin E heterozygosity or homozygosity (see page 327). Coinheritance of heterozygosity for $\beta^{\text{New York}}$ leads to more severe disease as the $\beta^{\text{New York}}$ chain has greater affinity than the normal β chain for the available α chains and haemoglobin New York is unstable [109]. Coinheritance with heterozygosity for a β chain variant can lead to the absence of detectable haemoglobin H (e.g. with haemoglobin Tak or haemoglobin Hope) or the presence of both haemoglobin H and haemoglobin Bart's (e.g. with haemoglobin J-Bangkok or haemoglobin Pyrgos) [162].

Haemoglobin Bart's hydrops fetalis and related conditions

Homozygosity for α^0 thalassaemia leads to a total absence of α chain synthesis so that no synthesis of haemoglobin F, A or A₂ can occur. The result is the clinical syndrome known as haemoglobin Bart's hydrops fetalis, first described in Indonesia in 1960 [163]; the designation ' α thalassaemia major' is sometimes used. This condition is most often seen in South-East Asia and southern China but there is a low but significant incidence in Greece, Turkey and Cyprus. Rare cases have been observed in Sardinia and in families of Indian and Pakistani ethnic origin. It has been estimated that in the UK, 20–30 pregnancies a year carry a risk of haemoglobin Bart's hydrops fetalis, mostly in people of South-East Asian origin.

Characteristic clinical features include severe anaemia, hepatosplenomegaly, cardiac failure, serous effusions and gross oedema of the fetus and placenta, although some fetuses are anaemic but not hydropic. Anaemia can be detected *in utero* by measurement of blood velocity in the middle cerebral artery, and may be identified from as early as the 12th week. There is hypoalbuminaemia (likely to be related to the marked extramedullary haemopoiesis in the liver). The severe anaemia and failure of oxygen delivery to tissues lead to abnormal organogenesis. Developmental abnormalities can include congenital cardiac abnormalities (atrial septal defect and patent ductus arteriosus), genital abnormalities (ambiguous genitalia,

hypospadias, undescended testes), terminal transverse limb defects, pulmonary hypoplasia and retarded brain growth [8, 164–166]. In one study developmental abnormalities were present in 64% of 58 infants with data available [164]. There is often growth retardation.

Haemoglobin Bart's hydrops fetalis is usually incompatible with extrauterine life. Some fetuses die *in utero* and others within a short time of birth. Rarely there is survival for a few days, even in the absence of treatment. Increasing number of fetuses have been 'rescued' by intrauterine or post-delivery transfusion [16, 164, 167, 168]; sometimes significant brain damage has already occurred, leading to cognitive and motor impairment [130], but this is not necessarily so [159]. In comparison with β thalassaemia major, there is earlier iron overload with more endocrine impairment and short stature [168].

Deletion of all four α genes but with one or both ζ genes being intact (e.g. --SEA/--SEA , --MED/--MED , --SEA/--THAI or --SEA/--FIL) leads to haemoglobin Bart's hydrops fetalis (Fig. 3.23). Exceptionally, the condition is due to uniparental disomy with two copies of an α^0 mutation being derived from either the mother or the father [169]. Almost all the haemoglobin present is haemoglobin Bart's, a haemoglobin with γ tetramers, which is unable to deliver oxygen to tissues since, like haemoglobin H, it has a very high oxygen affinity and lacks haem–haem interaction, leading to a hyperbolic rather than sigmoid oxygen dissociation curve. The remainder of the haemoglobin is largely haemoglobin Portland 1 ($\zeta_2\gamma_2$) which is capable of oxygen delivery to tissues and can keep the fetus alive into the third trimester. There may be small amounts of haemoglobin Portland 2 ($\zeta_2\beta_2$) and haemoglobin H. There is severe anaemia (consequent on a reduced rate of haemoglobin synthesis together with ineffective haemopoiesis and shortened red cell life span caused by precipitation of haemoglobin Bart's) and extramedullary haemopoiesis leading to hepatosplenomegaly. Because haemoglobin Bart's is a high affinity haemoglobin the functional effects of the anaemia are much greater than would be expected from the

Fig. 3.23 Fetus with haemoglobin Bart's hydrops fetalis. (With thanks to Dr Robyn Rodwell and by courtesy of the late Professor Harry Smith.)



haemoglobin concentration. At birth, the neonate is pale, faintly jaundiced, growth retarded and usually hydropic. There may be pulmonary hypertension and respiratory distress. There may also be subcutaneous blue nodules of haemopoietic tissue.

When there is homozygosity for a large deletion including the ζ genes as well as both α genes, no functional haemoglobin can be produced. The only haemoglobins synthesised are haemoglobin Bart's and a haemoglobin with tetrameric ϵ . The fetus dies early in gestation with consequent miscarriage. This syndrome can result from homozygosity for $--^{FIL}$ and $--^{THAI}$.

Rarely the phenotype of hydrops fetalis can result from heterozygosity for α^0 thalassaemia and severe non-deletional α^+ thalassaemia (e.g. $--/\alpha^{T\text{Saudi}}\alpha$, $--/\alpha^{QS}\alpha$ or $--/\alpha^{59\text{Gly}\rightarrow\text{Asp}}\alpha$). The disease phenotype may be more severe if the ζ locus is deleted as well as the $\alpha\alpha$ locus; this can be designated by the notation $-- --/\zeta (\alpha\alpha)^T$. Haemoglobin A and F are present in addition to haemoglobin Bart's, haemoglobin Portland 1 and haemoglobin H [170]. Proportions of the various haemoglobins are similar to those in haemoglobin H disease but the haemoglobin concentration during intrauterine life and at birth is lower. The genotype $--/\alpha^T\alpha$ more often results in haemoglobin H disease. The genotype $\alpha^{Adana}\alpha/\alpha^{Adana}\alpha$, which occurs in Indonesia, is associated with hydrops fetalis [59]. Homozygosity for haemoglobin Agrinio, which

has a very unstable α chain, can also be causative [171].

Women carrying a fetus with haemoglobin Bart's hydrops fetalis have an increased rate of pregnancy-related hypertension, severe anaemia, polyhydramnios and oligohydramnios and usually have a difficult delivery as the result of delivering a hydropic fetus and hydropic placenta [130, 164]; there is an increased rate of antepartum and postpartum haemorrhage and retained placenta and a high rate of caesarean section. Haemoglobin Bart's hydrops fetalis is often first detected by the identification of fetal anaemia on an ultrasound scan. If molecular diagnosis of α^0 thalassaemia is precluded by economic constraints, consideration should be given to ultrasound examination commencing early in pregnancy in potentially at-risk pregnancies in order to detect hydrops fetalis and inform discussions with parents before maternal complications occur.

When a fetus is rescued by intrauterine transfusion and subsequently maintained on a life-long transfusion regime similar to that used in β thalassaemia major (e.g. aiming for a pretransfusion Hb of $>90\text{ g/l}$), erythropoiesis is inadequately suppressed, non-functional haemoglobin H is present and iron overload occurs – attributed to increased gastrointestinal absorption as well as transfusion. More intensive treatment than for patients with β thalassaemia major is therefore needed in order to suppress erythropoiesis and prevent the

haemolysis and poor oxygen delivery that result from a haemoglobin H of 24–64% [169]. A pretransfusion Hb of >100 g/l and a haemoglobin H <15% have therefore been recommended [167, 172] but a better approach, given that haemoglobin H is non-functional, is to aim at a pretransfusion functional Hb of 106 g/l and a haemoglobin H less than 16.1% [173]. Regular exchange transfusion is an alternative [174]. Splenectomy is associated with a high rate of thromboembolic complications [174].

Laboratory features

A fetus with haemoglobin Bart's hydrops fetalis has severe anaemia (Hb usually 30–80 g/l, occasionally up to 100 g/l or even higher) and striking anisocytosis and poikilocytosis (including target cells and elongated cells). Erythrocytes appear large but markedly hypochromic. There is a reticulocytosis. Circulating nucleated red cells are greatly increased, a condition designated erythroblastosis fetalis (Fig. 3.24). The MCH and MCHC are greatly reduced but MCV can be normal.

Haemoglobin electrophoresis (Fig. 3.25) and HPLC (Fig. 3.26) show haemoglobin Bart's (70%–100%) and sometimes smaller amounts of haemoglobin Portland 1 (usually around 10–20%), haemoglobin Portland 2 and haemoglobin H. Tetramers of δ chain have also been detected.

Diagnosis

Following delivery of a hydropic fetus, the diagnosis of haemoglobin Bart's hydrops fetalis can be made from the haematological and electrophoretic/chromatographic characteristics. Sometimes the condition is diagnosed *in utero* as a result of ultrasound examination, which can detect an enlarged placenta, increased cardiothoracic ratio and increased rate of blood flow in the middle cerebral artery. It is desirable that this condition is identified early in pregnancy by an antenatal screening programme that allows the identification of all women at risk of α^0 thalassaemia trait followed, when both partners are at risk of α^0 thalassaemia trait, by

DNA analysis. When both parents are found to have α^0 thalassaemia trait (or have haemoglobin H disease), the potential benefits of prenatal diagnosis using fetal DNA analysis should be discussed. Fetal tissue for diagnosis can be obtained by chorionic villous sampling in the first or second trimester, from amniotic fluid obtained by amniocentesis in the second trimester and by fetal blood sampling in the second trimester. Management options are termination of pregnancy or intrauterine transfusion with the knowledge that life-long transfusion therapy will be required and the risk of disability is high. *In utero* haematopoietic stem cell transplantation (HSCT) has been attempted unsuccessfully on three occasions, with little evidence at autopsy of effective engraftment [175]. A clinical trial of *in utero* HSCT for haemoglobin Bart's hydrops fetalis is ongoing in the USA, with protocols for *in utero* gene therapy being developed.

Coinheritance of α thalassaemia and other haemoglobinopathies

As commented on above, coinheritance of α^0 thalassaemia and an unstable α chain variant can cause haemoglobin H disease. Coinheritance of α thalassaemia and an α chain variant haemoglobin will increase the percentage of the variant and if this has undesirable characteristics, e.g. high affinity or methaemoglobinaemia, the effects will be aggravated. Coinheritance of α thalassaemia and a β chain variant may decrease (e.g. haemoglobin S, C or E) or increase (e.g. haemoglobin J-Baltimore or J-Iran) the percentage of the variant, depending on the relative affinities of the variant and normal β chains for the reduced numbers of α chains. The effects of β thalassaemia genes are ameliorated by α thalassaemia (see later).

β thalassaemias

The beta or β thalassaemias are a group of conditions resulting from a reduced rate of synthesis of β globin. More than 380 genetic variants causing β thalassaemia have been recognised, occurring in a wide range of ethnic groups

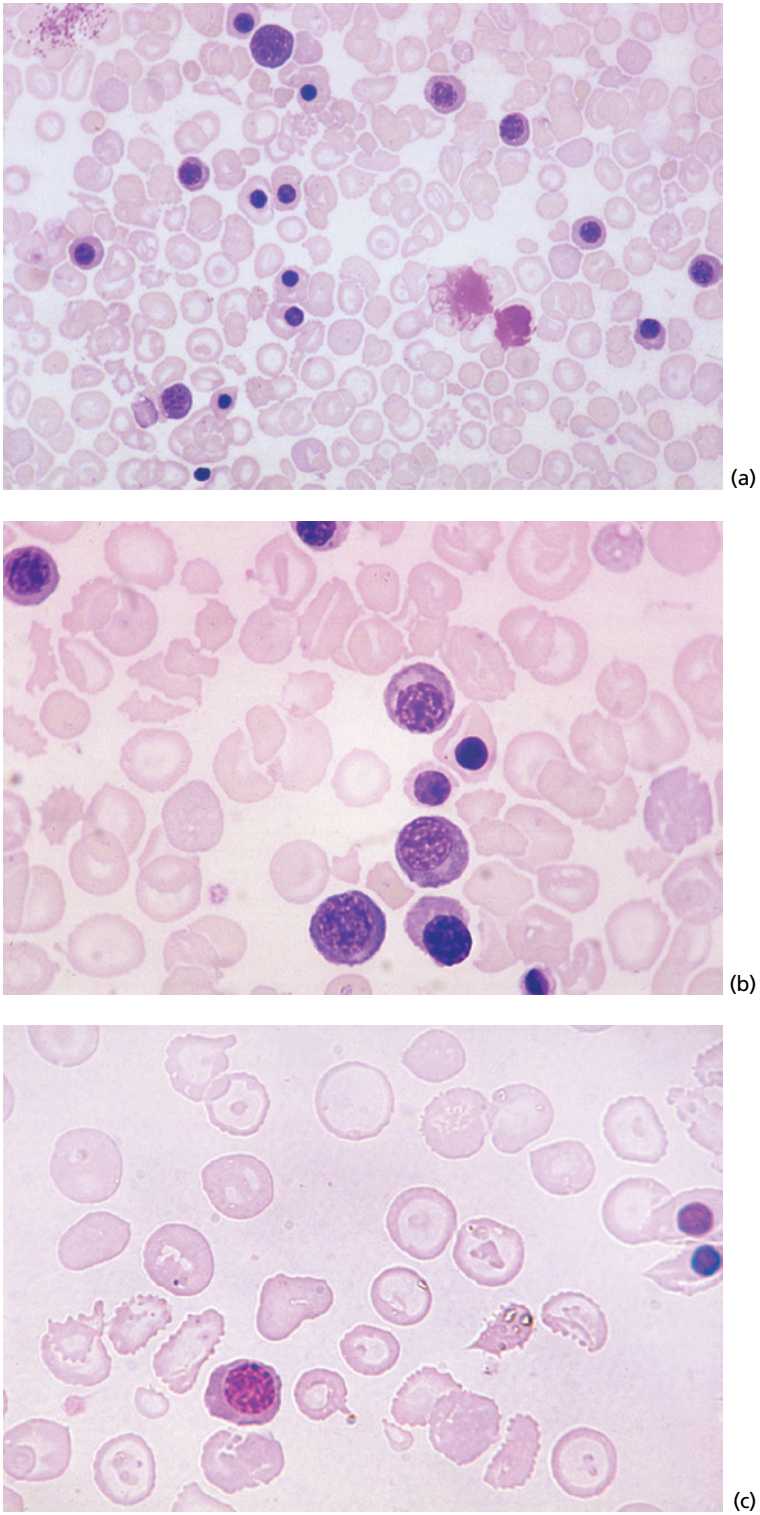


Fig. 3.24 Blood film in two cases of haemoglobin Bart's hydrops fetalis showing a striking increase in nucleated red blood cells: (a) MGG $\times 40$; (b) MGG $\times 100$ (with thanks to Professor Mary Frances McMullin); (c) MGG $\times 100$ (by courtesy of the late Professor Harry Smith).

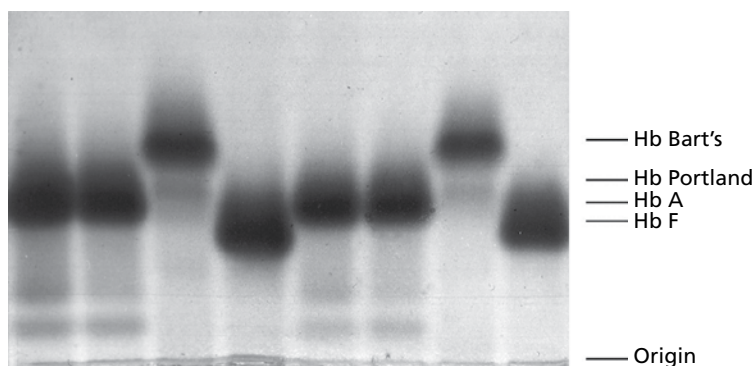


Fig. 3.25 Haemoglobin electrophoresis in haemoglobin Bart's hydrops fetalis, third and seventh patterns from left, showing a major haemoglobin Bart's band and a minor haemoglobin Portland band. (With thanks to Dr Helen Dodsworth.)

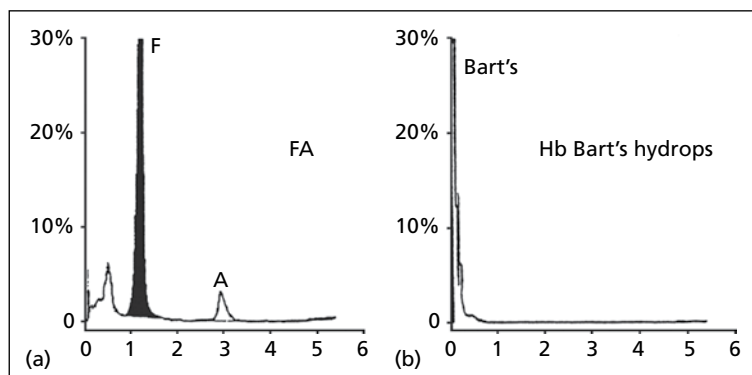


Fig. 3.26 HPLC chromatogram (Bio-Rad Variant) in (a) a normal neonate showing post-translationally modified haemoglobin F, haemoglobin F (black) and haemoglobin A and (b) in a neonate with haemoglobin Bart's hydrops fetalis showing haemoglobin Bart's only. ([91]/with permission of Oxford University Press.)

(see Table 3.5), including more than 350 variants involving a small number of nucleotides and at least 18 large deletions. β thalassaemia is common throughout the world, apart from in northern Europe and indigenous populations in the Americas and Australia. It is recognised but rare in the indigenous British population [176] and in other northern European populations. The severity of the defect is very variable. Since normal individuals have two allelic β globin genes, β thalassaemia can exist in a heterozygous or homozygous state. Since there are a large number of β thalassaemia mutations, compound heterozygosity can also occur with the individual having two different β globin variants but no normal β gene.

β thalassaemia mutations are divided into two broad categories, β^0 (beta zero) thalassaemia and β^+ (beta plus) thalassaemia. In β^0 thalassaemia there is either an abnormal gene that is not expressed or, less often, most or all of the gene is deleted. If β^0 thalassaemia occurs in the homozygous or compound heterozygous state

($\beta^0\beta^0$) there is a total lack of β chain production and a total failure to produce haemoglobin A. In β^+ thalassaemia there is reduced but not absent expression of the abnormal β gene so that when β^+ thalassaemia alleles are inherited from both parents, there is some production of haemoglobin A. There are a large number of different β^+ thalassaemia mutations with the severity of the defect in chain synthesis varying from mild to severe. People with two β^+ thalassaemia alleles therefore have disease varying from mild to very severe. Compound heterozygotes for β thalassaemia may have two different β^0 thalassaemia genes, two different β^+ thalassaemia genes or both a β^0 and a β^+ thalassaemia gene.

β thalassaemia usually results from a variant occurring in or near the β gene. A smaller proportion of cases result from deletion, either of the β gene itself or of controlling sequences 5' to the gene (i.e. upstream from the gene). β thalassaemia can also result from mutations that lead to structural abnormalities of the β globin chain; the mechanism may be either reduced rate of β

chain production or production of a very unstable β chain or a very unstable haemoglobin. Haemoglobin E is the most prevalent example of a structurally abnormal haemoglobin in which the mutated β globin is synthesised at a reduced rate, in this instance as a result of activation of a cryptic splice site. It is sometimes referred to as a thalassaemic haemoglobinopathy. Haemoglobin Malay, another variant haemoglobin with activation of a cryptic splice site, represents 15% of thalassaemia alleles in Malaysia and 16% in southern Thailand and also occurs in China and Indonesia [177]; it is likely to be frequently unidentified since it cannot be distinguished from haemoglobin A by electrophoresis or chromatography. Haemoglobin North Shore and haemoglobin Vicksburg are less common variant haemoglob-

ins associated with a β thalassaemia phenotype [178]. Haemoglobin Dhohar, found in Oman, is associated with a thalassaemic phenotype [179]. It results from the coexistence of two mutations, one causing a structural alteration and the other, which creates an alternative splice site, being responsible for the thalassaemic features [179]. Heterozygotes have 9–21% of the variant haemoglobin and typical thalassaemic red cell indices [179]. Homozygotes have the clinical features of thalassaemia major or severe thalassaemia intermedia [179]. Mutations that can result in β thalassaemia and the phenotype usually associated with each are summarised in Table 3.7 [9, 180–191] and the sites of mutations leading to β^0 and β^+ thalassaemia respectively are summarised in Figs. 3.27 and 3.28. Rarely there are two thalassaemia

Table 3.7 Types of mutation that can result in the phenotype of β thalassaemia (the numbers of mutations given should be regarded as approximate since new mutations continue to be described) [9, 180–191].

Type of mutation	Consequence	
Deletional		
Large deletion involving the β gene (at least 15 reported, only that occurring in Sind and Punjabi populations is common)	Absent transcription, unusually high haemoglobin A ₂ in heterozygotes	β^0 thalassaemia
Small deletion 5' of the β gene (at least three reported)	Reduced transcription	β^+ thalassaemia
Non-deletional		
Insertion of a transposable element into IVS2 (One reported)	Reduced transcription	β^+ thalassaemia
Mutations in promoter sequences, CACCC, CCAAT or TATA box (at least 26 reported)	Reduced transcription, increased transcription of γ and δ	Silent β , mild (β^{++}) thalassaemia or β^+ thalassaemia
Mutation in 5' untranslated region near CAP site (at least eight reported)	Reduced transcription and translation and instability of mRNA	Silent or mild (β^{++}) thalassaemia
Mutation of initiation codon (at least nine reported)	Absent transcription	β^0 thalassaemia (more severe than most other β^0 thalassaemia)
RNA splicing mutations – involving invariant nucleotides of either donor or acceptor site (at least 27 reported)	Absence of properly spliced mRNA	β^0 thalassaemia
RNA splicing mutations – involving nucleotides flanking splice junctions (consensus sequences) (at least 12 reported)	Inefficient splicing of mRNA	Silent, mild (β^{++}) thalassaemia or β^+ thalassaemia or occasionally, β^0 thalassaemia
(Continued on p. 134.)		

(Continued on p. 134.)

Table 3.7 Continued.

Type of mutation	Consequence	
RNA splicing mutations – activation of cryptic splice site in an intron or an exon with or without an alteration in the coding sequence (at least 12 reported)	Aberrant mRNA is produced in addition to normal mRNA; sometimes a structurally abnormal β chain is produced, which may be highly unstable	Mild (β^{++}) thalassaemia, β^+ thalassaemia. β^0 thalassaemia or, occasionally, dominant β thalassaemia; haemoglobin E, haemoglobin Malay, Hb Knossus
Mutation interfering with polyadenylation and therefore mRNA cleavage (at least seven nucleotide substitutions and at least three small deletions reported)	Unstable elongated RNA transcript (plus some normal transcript)	β^+ or mild (β^{++}) thalassaemia
Other mutations interfering with mRNA processing (at least four reported)	Abnormal processing of messenger RNA	Silent, mild (β^{++}) or β^+ thalassaemia
Premature termination codon consequent on alteration of a single nucleotide (at least 16 reported) or on a frameshift mutation (at least 72 reported, including one large insertion in exon 2 and other deletions and insertions)	With absent translation (exon 1 and 2 mutations) or translation of aberrant mRNA (exon 3 mutations) leading to a very unstable truncated β chain	β^0 thalassaemia (exon 1 and 2 mutations) or dominantly inherited β thalassaemia (exon 3 mutations)
New sense mutation, alteration of STOP codon to a coding sequence		Hb Zunyi (dominant β thalassaemia with reduced Hb A ₂)
Other 'thalassaemic haemoglobinopathies' caused by a single nucleotide change	With reduced transcription of an abnormal mRNA	Hb North Shore, Hb Vicksburg
	With transcription of abnormal RNA coding for a very unstable β chain	Hb Indianapolis, Hb Geneva
'Thalassaemic haemoglobinopathies' associated with mutation of the termination codon to a coding sequence ('anti-termination' mutation)	Synthesis of an elongated mRNA and β chain	Hb Tak, Hb Cranston, Hb Saverne
Haemoglobin Lepore*	$\delta\beta$ fusion gene with reduced rate of synthesis of aberrant $\delta\beta$ chain	Hb Lepore Boston, Hb Lepore Baltimore, Hb Lepore Hollandia
Certain unstable haemoglobins	Marked instability of haemoglobin leading to rapid post-translational degradation	Hb Showa-Yakushigi Hb K Woolwich
Mutation in the <i>GATA1</i> gene, a gene at Xp11.23 encoding an erythroid transcription factor	Reduced transcription	Thalassaemia with thrombocytopenia and (in one patient) congenital erythropoietic porphyria
Mutation in the <i>ERCC2</i> (previously <i>XPD</i>) gene at 19q13.32, which encodes a component of the general transcription factor, TFIIH	Reduced transcription	Thalassaemia with trichothiodystrophy
Mutation in <i>SUPT5H</i> †	Transacting factor	β thalassaemia [190]

* Haemoglobin Lepore can be regarded as a $\delta\beta^+$ thalassaemia.† Can also result in a raised haemoglobin A₂ but with normal red cell indices [191].

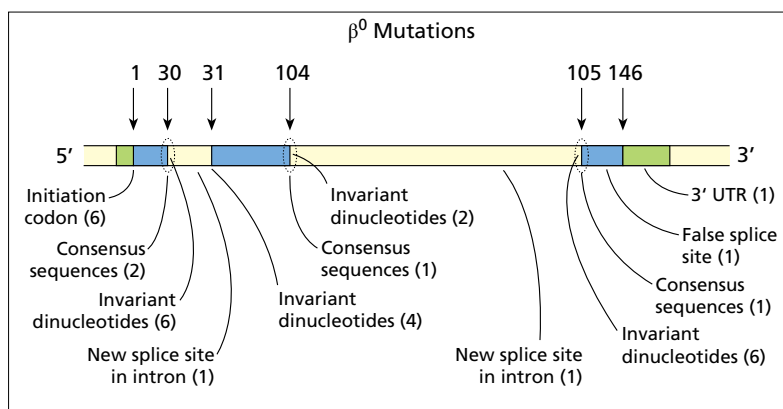


Fig. 3.27 Sites of some mutations giving rise to β^0 thalassaemia, including dominant thalassaemia. The numbers above the chromosome represent the codons. Introns are shown in blue and the 5' and 3' untranslated regions (UTR) in green. The dotted lines are a diagrammatic indication of the consensus sequences flanking the invariant nucleotides at the splice sites. In addition to the mutation sites shown, there are now several hundred reported mutations including nonsense mutations (some dominant), frameshift mutations, unstable variants (some dominant), and insertions or deletions of triplet codons (some dominant).

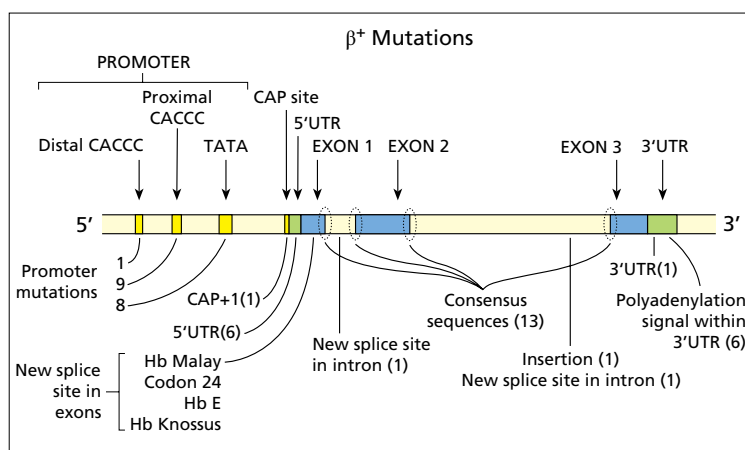


Fig. 3.28 Sites of some mutations giving rise to β^+ thalassaemia. Introns are shown in blue, the 5' and 3' UTR in green and important promoter sequences in yellow. The dotted lines are a diagrammatic indication of the consensus sequences flanking the invariant nucleotides at the splice sites.

mutations on one chromosome, as in a patient with both IVS2 654 C \rightarrow T and 3'UTR nt 1570T \rightarrow C [192]. The great majority of cases of β thalassaemia result from mutations in or near the β globin gene (*HBB*). However there are occasional cases with no detectable abnormality in the β globin cluster and in which inheritance is not linked to the cluster [193, 194]. Such cases can result from a mutation in a gene encoding a *trans*-activating factor: in *ERCC2* (previously

XPD), encoding a component of a general transcription factor, TF11H; in *GATA1*, encoding an erythroid-specific transcription factor; or in *ASH1L*, encoding a histone methyltransferase (see Table 1.4). In the case of a *GATA1* mutation, the β thalassaemia trait can be associated with thrombocytopenia [187].

β thalassaemia heterozygosity, also referred to as β thalassaemia trait or being a β thalassaemia carrier, is usually clinically

asymptomatic, a condition that may also be referred to as β thalassaemia minor. Homozygosity or compound heterozygosity for β thalassaemia usually leads to a clinically severe phenotype, referred to as β thalassaemia major, in which the individual is dependent on blood transfusion. The term 'thalassaemia intermedia' indicates that the clinical features of a case are intermediate between those of β thalassaemia minor and of β thalassaemia major; the individual is symptomatic but although he or she may need occasional blood transfusions, these are not essential to maintain life. The clinical conditions designated thalassaemia intermedia cover a genetically heterogeneous group of disorders, of very variable severity, including homozygosity or compound heterozygosity for mild β^+ thalassaemia and heterozygous β thalassaemia aggravated by coexisting heterozygosity or homozygosity for $\alpha\alpha$. β thalassaemia mutations that are characteristically associated with clinical abnormalities in heterozygotes are referred to as dominant β thalassaemia (see page 151). Although, by definition, blood transfusion is not essential to maintain life in thalassaemia intermedia, patients with more severe disease may be treated electively with blood transfusion in order to improve quality of life. To a large extent, the distinction between severe thalassaemia intermedia and thalassaemia major is arbitrary and depends on the clinical decision to start regular blood transfusions rather than any underlying genetic or pathophysiological differences. Reflecting this, the term non-transfusion-dependent thalassaemia (NTDT) is increasingly used instead of thalassaemia intermedia, and transfusion-dependent thalassaemia (TDT) is used instead of thalassaemia major.

β thalassaemia trait

β thalassaemia trait or heterozygosity for β thalassaemia is often completely asymptomatic and was previously referred to as β thalassaemia minor. However, a controlled study in Sri Lanka found that β thalassaemia heterozygotes were significantly more likely to have symptoms of

anaemia such as lethargy, fatigue and dizziness [195]. They also had more febrile episodes, suggesting an increased susceptibility to infection [195]. In conditions of haemopoietic stress, for example during pregnancy or during intercurrent infections, the patient may become anaemic and even require blood transfusion. A large retrospective study in Sicily found an association with cholelithiasis, kidney disease, cirrhosis and mood disorders [196]. The risk of gallstones is further increased by coinheritance of Gilbert syndrome [197]. Renal tubular dysfunction is seen in a significant minority of patients [198]. There appears to be a higher incidence of rheumatoid arthritis [199]. Occasional patients have palpable splenomegaly and ultrasonography shows that splenic volume is increased in comparison with controls [200].

Serum ferritin is higher in β thalassaemia heterozygotes [201]. Iron overload can occur, particularly as a result of long continued inappropriate administration of iron [202]. The clinicopathological features of hereditary haemochromatosis are aggravated by β thalassaemia trait and it is possible that the risk of iron overload is increased in individuals with an *HFE* genotype associated with a mild risk of haemochromatosis [203]. A single case report suggests that iron overload may also be aggravated in patients with a *TRF2* mutation [204]. In patients with chronic hepatitis C infection, β thalassaemia heterozygosity is associated with more hepatic iron deposition and more fibrosis [205]. Similarly, there is an association with hepatic siderosis and fibrosis in patients with non-alcoholic fatty liver disease [206].

Several studies have found that β thalassaemia heterozygosity protects against myocardial infarction in men [207, 208] or protects against ischaemic heart disease [209] or severe coronary artery disease [210] but in another study protection against coronary artery disease was not found [211]. β thalassaemia trait is associated with a lower prevalence of hypertension and, in men, with protection against ischaemic stroke [212]. It may also protect against peripheral vascular disease [213]. The plasma concentration of cholesterol and low density lipoproteins is reduced, even in subjects

with familial hypercholesterolaemia, while triglycerides and high density lipoproteins are normal [214, 215]. Beneficial effects on vascular disease could be attributable to reduced viscosity as well as effects on lipids. Epidemiological studies in Sardinia (previously a malaria area) and Melanesia have shown β thalassaemia to have an inverse relationship with altitude and the prevalence of malaria, consistent with a protective effect [216]. Evidence from Sri Lanka also supports a protective effect [74].

There are conflicting data on the prevalence of iron deficiency during pregnancy in women with β thalassaemia trait.

As β thalassaemia trait is very common, it is frequently coinherited with other red cell abnormalities and may interact with these to produce an unusual or unexpectedly severe clinical picture. For example, two patients have been reported in whom coinheritance with South-East Asian ovalocytosis led to significant iron overload [217], and coinheritance with a *PIEZO1* variant led to symptomatic disease with anaemia, splenomegaly and haemolysis [218].

Laboratory features

The blood count characteristically shows a normal or slightly reduced Hb, elevation of the RBC and reduction of the MCH and MCV. The MCHC is usually normal when measured by impedance counters (e.g. Coulter or Sysmex instruments) but may be reduced when measured by Siemens light scattering instruments. The red cell distribution width (RDW), a measurement that reflects red cell anisocytosis, is usually normal. Mean values for haematological variables (Hb, MCV and MCH) differ significantly between β^+ and β^0 thalassaemia trait but there is overlap [219, 220]. An MCV of less than 67 fl and an MCH of less than 21 pg give a reasonable separation of β^0 from β^+ thalassaemia [220]. The reticulocyte count was elevated in about a quarter of patients in one series [221] and in another mean counts were 3% and $150 \times 10^9/l$ [222]. The steady state Hb is lower if there is coinheritance of G6PD deficiency, a mean Hb of 125.5 g/l being

observed in comparison with a mean of 136.1 for uncomplicated β thalassaemia trait [223]; the reduction in MCV and MCH was marginally less when G6PD deficiency coexisted. In another study the MCV was again somewhat higher in subjects with G6PD deficiency, the mean difference being about 3 fl [149]. The haematological abnormality is less if there is coinheritance of α thalassaemia (see later). The Hb falls in pregnancy since plasma volume rises more than the red cell mass [224]. However, an Hb of less than 80–90 g/l suggests a complicating factor [224]. During pregnancy, the MCV rises, on average, by about 2 fl, in comparison with a rise averaging about 4 fl in haematologically normal subjects [225]. The percentage of reticulocytes may be slightly elevated. Reticulocyte indices (Advia® 120 Hematology System) are significantly different from those of iron deficiency, MCVr (reticulocyte mean cell volume), CHr (mean reticulocyte content of haemoglobin) and MCHCr (MCHC of reticulocytes) are all significantly lower, with the CHr showing no overlap [226].

The characteristic red cell indices have been used in a number of formulae designed to separate cases of iron deficiency from cases of β thalassaemia trait [227–236]; these were tabulated in the first edition of this book [237]. Although these formulae may be of some value in separating uncomplicated cases into two diagnostic categories, they are unreliable in children and during pregnancy and are of no help in patients who have both β thalassaemia trait and iron deficiency. Patients who are under treatment for iron deficiency and those who have polycythaemia vera complicated by iron deficiency can also have results more suggestive of thalassaemia trait than of iron deficiency. In a study of randomly selected adult patients with mild microcytosis none of the four most popular formulae was found to be more effective than the MCV (cut-off point <72 fl) in distinguishing thalassaemia trait from other conditions [236]. These various formulae may be useful in indicating the most likely diagnosis but they are not of use when it is necessary to either make or exclude a definite diagnosis of β thalassaemia trait. Their use is therefore not recommended.

Ferrokinetic and red cell survival studies indicate an increase in ineffective erythropoiesis and a shortened red cell life span [238].

It should be noted that when a patient with β thalassaemia trait develops megaloblastic anaemia or significant liver disease the MCV and the MCH may rise into the normal range. The same will occur with administration of drugs such as zidovudine or hydroxycarbamide but in these patients there is usually a pretreatment blood count showing characteristic red cell indices.

The blood film varies from almost normal, with only mild microcytosis, to markedly abnormal (Fig. 3.29). Abnormal features, in addition to microcytosis, include anisocytosis, hypochromia and poikilocytosis. Individuals with a more severe phenotype may have prominent basophilic stippling, target cells and small

numbers of irregularly contracted cells. Elliptocytes are generally more characteristic of iron deficiency than of thalassaemia trait but some thalassaemic individuals have prominent elliptocytes. Target cells and basophilic stippling are generally more common in β thalassaemia than in iron deficiency. Anisochromasia (i.e. variation in the degree of haemoglobinisation from one cell to another) is characteristic of iron deficiency and is not usually a feature of uncomplicated β thalassaemia trait. Patients with β thalassaemia trait who have required splenectomy for any reason may have a much more bizarre blood film (Fig. 3.30).

Zinc protoporphyrin is elevated in two-thirds of patients; values tend to be lower than in iron deficiency although there is some overlap [89]. Serum soluble transferrin receptor concentration

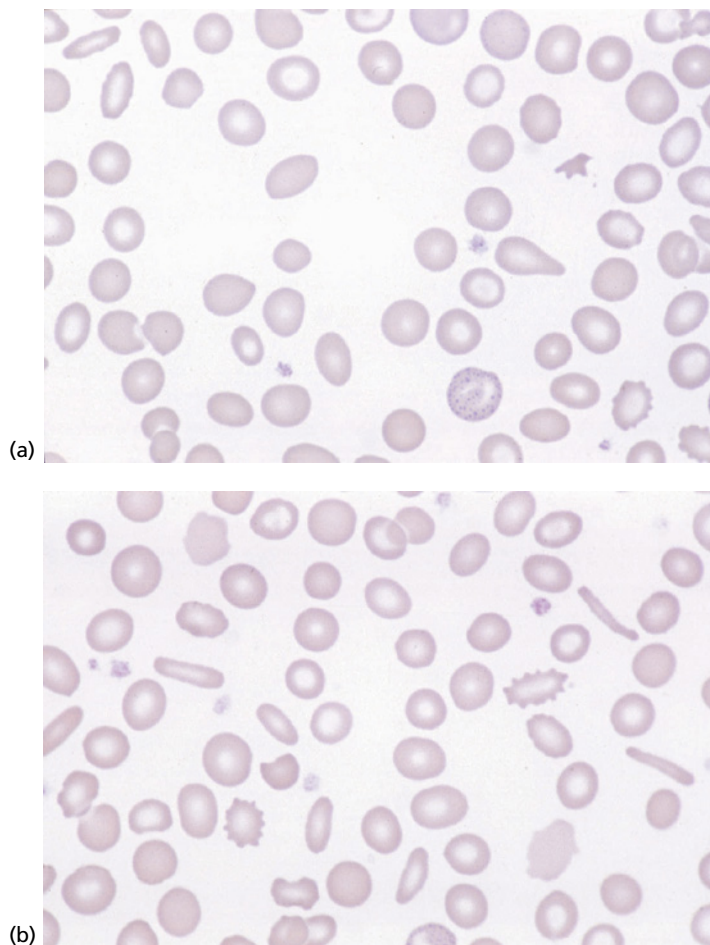
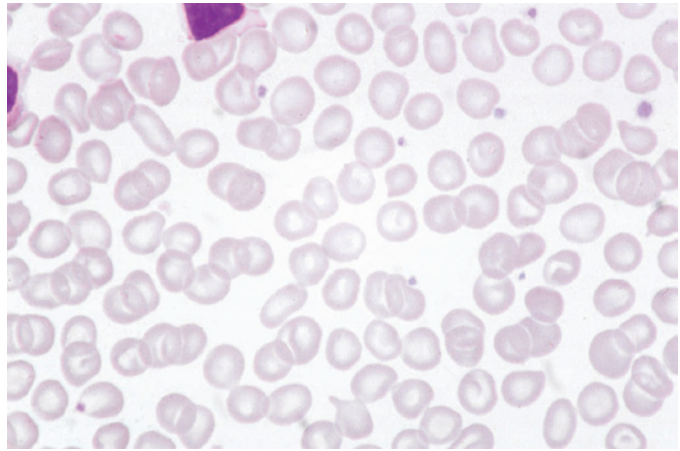
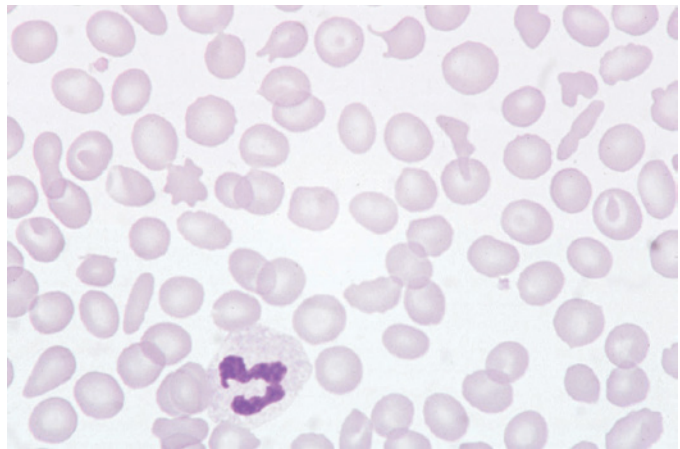


Fig. 3.29 Blood films from three patients with β thalassaemia trait showing the range of abnormalities observed: (a) blood film showing microcytosis, anisochromasia, a tear drop poikilocyte and basophilic stippling; the red cell indices were RBC $4.41 \times 10^{12}/l$, Hb 140 g/l, Hct 0.42, MCV 69 fl, MCH 23.2 pg, MCHC 334 g/l; (b) from the same patient showing unusually prominent elliptocytes;



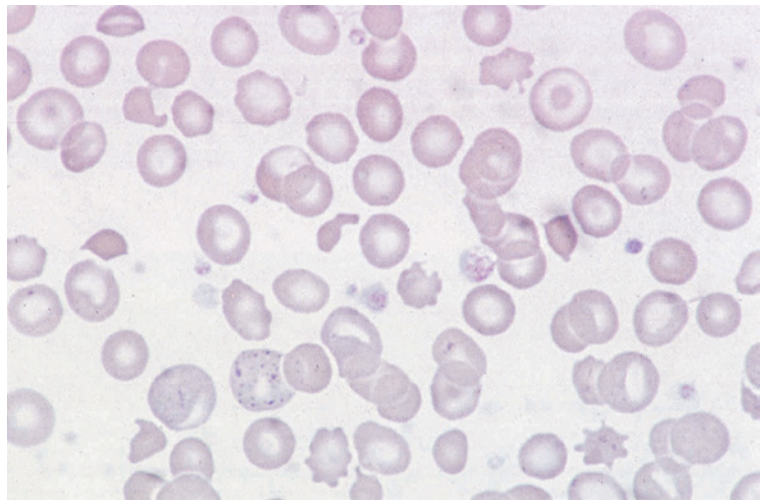
(c)

Fig. 3.29 *Continued.* (c) from a patient with RBC $7.3 \times 10^{12}/l$, Hb 143 g/l, Hct 0.43, MCV 59 fl, MCH 19.9 pg, MCHC 328 g/l, haemoglobin A₂ 5.6%, haemoglobin F 0.6%; (d) from a patient with β^0 thalassaemia trait demonstrated by family studies. MGG $\times 100$.



(d)

Fig. 3.30 Blood film from a patient with β thalassaemia trait who had been splenectomised because of a lymphoma involving the spleen, showing very abnormal red cell morphology. MGG $\times 100$.



is increased. Pyrimidine 5' nucleotidase activity is reduced to levels comparable with those seen in heterozygotes for inherited deficiency [239]. Red cell survival is normal [240].

In subjects with G6PD deficiency, levels are higher in those with β thalassaemia trait when expressed per gramme (g) of haemoglobin [149]. However, it should be noted that the results of assays for G6PD are altered by coexisting β thalassaemia trait, depending on how they are expressed. One study showed that when G6PD levels were expressed in terms of g of haemoglobin or ml of red blood cells, concentration appeared to be increased in individuals with β thalassaemia trait, whereas if expressed in terms of number of red cells, concentration was normal [223]. In another study results were higher when expressed per g of haemoglobin and to a lesser extent when expressed per number of red cells [149].

The bone marrow aspirate (Fig. 3.31) shows increased cellularity as a result of erythroid hyperplasia. Some erythroblasts show defective haemoglobinisation and cytoplasmic vacuolation. An iron stain may show heavy siderotic granulation. Incubation of bone marrow with methyl violet shows small numbers of erythroblasts with α chain inclusions [224]. Ultrastructural examination shows α chain precipitates in a small proportion of late erythroblasts, accompanied by evidence of dyserythropoiesis such as duplication

of the nuclear membrane (Fig. 3.32a), autophagic vacuoles (Fig. 3.32b), myelin figures, glycogen accumulation and iron-laden mitochondria [241]. The presence of α chain precipitates correlates with reduced protein synthesis [241].

In the neonatal period, babies with β thalassaemia trait, in contrast to those with α thalassaemia trait, have a normal Hb and normal red cell indices. Differences from normal start to appear around the age of three months and the blood film may then be abnormal (Fig. 3.33). By the age of six months there is no appreciable overlap in red cell indices between those with and without β thalassaemia trait.

Diagnosis

Diagnosis rests on detection of an increased haemoglobin A_2 percentage (Figs 3.34 and 3.35; see also Fig. 2.7) [242, 243]. The increased haemoglobin A_2 percentage is due to an absolute rather than merely a relative increase in δ chain synthesis [224], for two reasons. An excess of α chains favours formation of haemoglobin A_2 ; in addition, some promoter mutations are associated with an increased rate of δ globin synthesis. Haemoglobin A_2 tends to be higher in β^0 than in β^+ thalassaemia but there is no clear separation [220]. Other inherited and acquired causes of a high haemoglobin A_2 percentage that should be considered in the differential diagnosis are

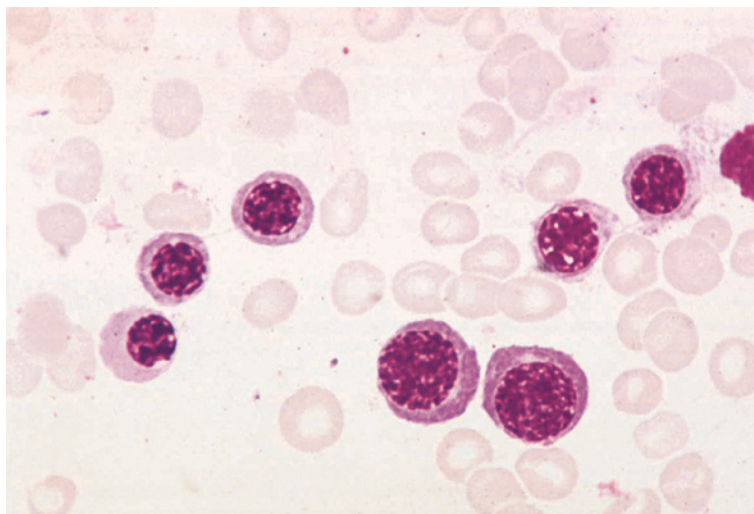
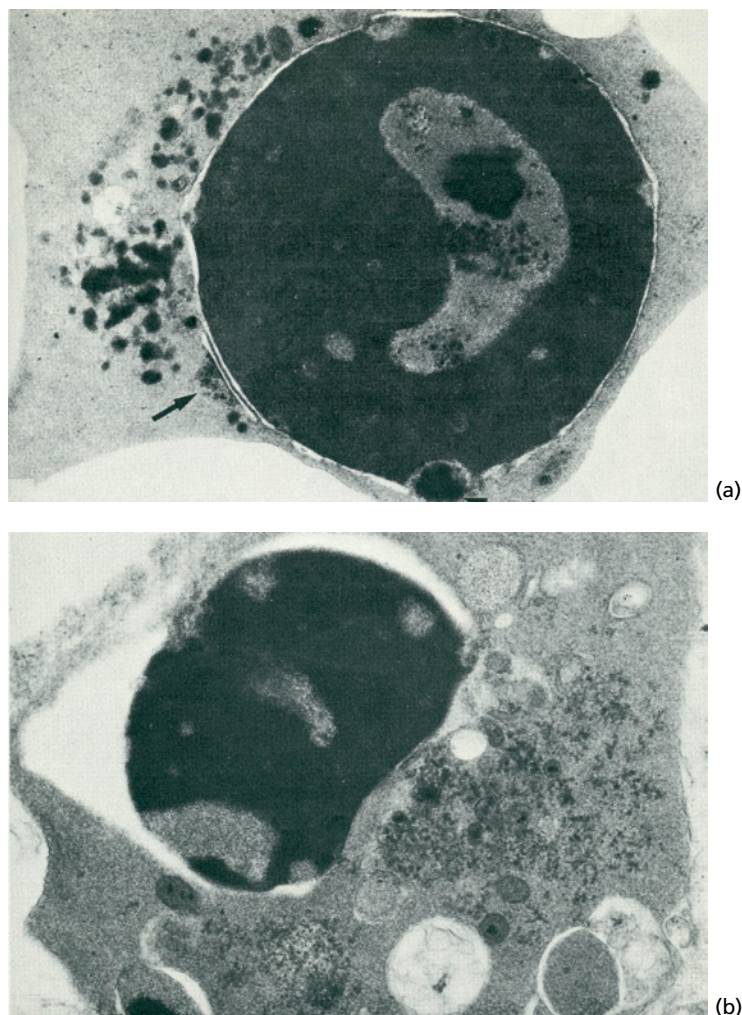


Fig. 3.31 Bone marrow aspirate in β thalassaemia trait showing mild dyserythropoiesis. MGG $\times 100$.

Fig. 3.32 Ultrastructural examination of the bone marrow in β thalassaemia trait showing α chain precipitates and dyserythropoiesis: (a) cytoplasmic and intranuclear α chain precipitates with duplication of the nuclear membrane in association with some of the precipitates (arrow); (b) small masses of cytoplasmic α chain precipitates associated with dilation of the space between the two layers of the nuclear membrane and several autophagic vacuoles. (By courtesy of the late Professor Sunitha N. Wickramasinghe and by permission of the British Journal of Haematology.)



shown in Tables 3.8 [244–258] and 6.3. The haemoglobin A_2 increases to above normal levels in the first few months of life (Fig. 3.36).

The relatively frequent occurrence of a variant haemoglobin A_2 , designated A_2' (previously B_2), must be considered when seeking to diagnose β thalassaemia trait. This variant A_2 has the same retention time on HPLC as haemoglobin S and thus, if haemoglobin S is absent, it is readily identified (Fig. 3.37). In one study haemoglobin A_2' was found in about 4% of samples [259]. It is found mainly in those of African ancestry, in whom prevalence is 1–2%, but has also been reported from Oman [260]. There are also haemoglobin A_2 variants that are present at a

low level as a result either of a reduced rate of synthesis or instability; nevertheless, the sum of A_2 plus A_2' variant may reach diagnostic levels [261]. In individuals with haemoglobin G-Philadelphia, haemoglobin G_2 elutes in the S window [259]. In seeking to make a diagnosis of β thalassaemia trait, haemoglobin A_2' and any other variant A_2 (whether resulting from a variant δ chain or from a variant α chain) must be added to normal haemoglobin A_2 and the total of the two used to determine whether 'haemoglobin A_2' is elevated.

A proportion of cases, around a third to a half, also have an increased proportion of haemoglobin F. Usually this is not more than 2–3% but

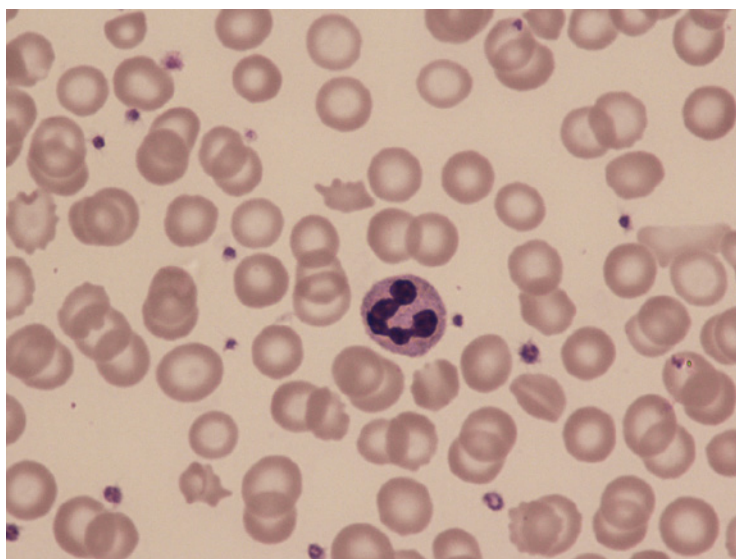


Fig. 3.33 Blood film of a three-month-old baby with β thalassaemia trait showing hypochromia, microcytosis and poikilocytosis. MGG $\times 100$.

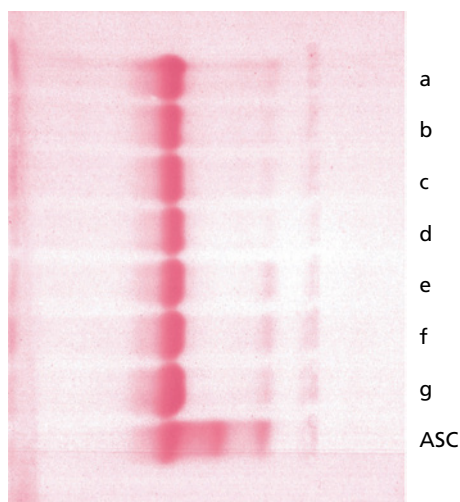


Fig. 3.34 Haemoglobin electrophoresis on cellulose acetate at alkaline pH showing increased haemoglobin A_2 in two patients with β thalassaemia trait (lanes e and f); ASC is a control sample containing haemoglobins A, S and C. Haemoglobin A_2 has the same mobility at C and E.

may be higher with deletional β^0 thalassaemia with levels of 4–11% [262].

In screening populations for β thalassaemia it is possible either to measure the haemoglobin

A_2 percentage in everyone or to screen by the red cell indices and test only those with an MCV or MCH below a certain cut-off point. An MCH of less than 27 pg is an indication to quantitate haemoglobin A_2 . Screening by the MCV may be less satisfactory as there is more variation in values between different instruments and with some instruments the measured MCV rises with storage of the blood sample [263]. In one study an MCH of less than 27 pg was found to be marginally more sensitive than an MCV of less than 80 fl [264]. In developing countries, where well calibrated automated instruments suitable for the measurement of red cell indices may not be available, osmotic fragility can be used for initial screening. The most effective screening strategy will depend on the prevalence of β thalassaemia trait in the target population. The cells of β thalassaemia trait (and of α thalassaemia trait, iron deficiency and some haemoglobinopathies) are more resistant to lysis in hypotonic solutions than normal cells (see page 416). Use of a single-tube visual osmotic fragility test reduces the number of samples that have to be referred to a central laboratory for definitive diagnosis. However, it should be noted that the sensitivity of the

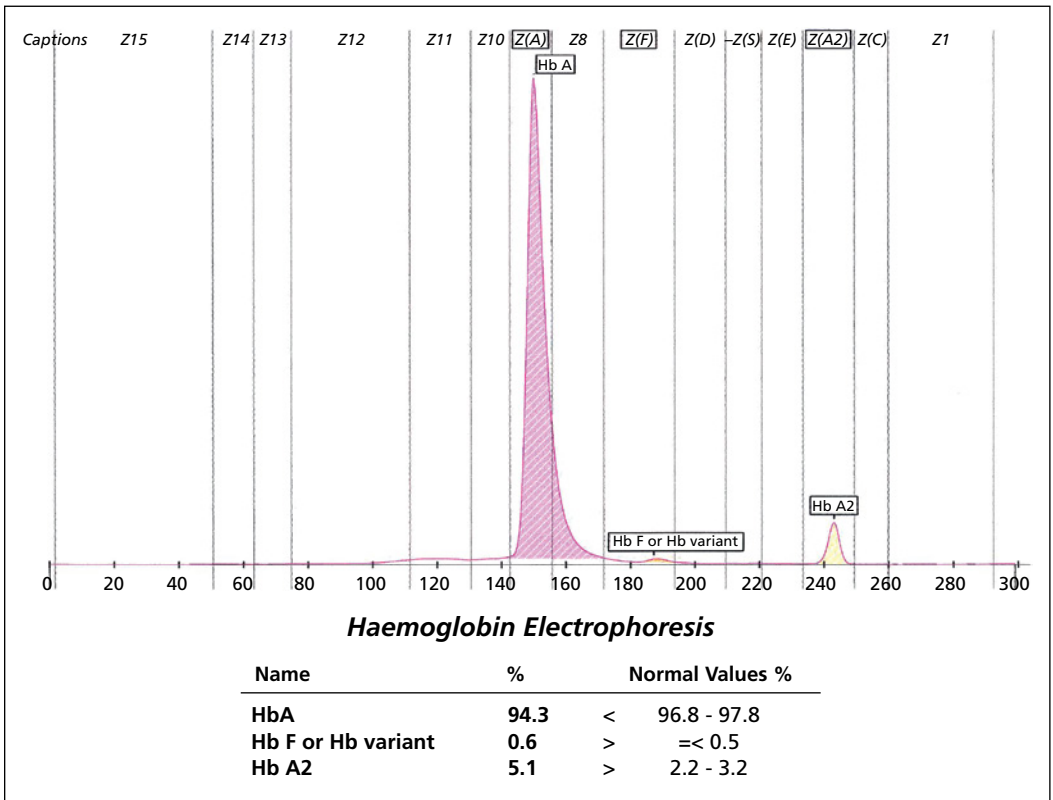


Fig. 3.35 Capillary electrophoresis, electropherogram (Sebia Capillarys 3) showing increased haemoglobin A₂.

osmotic fragility test is reduced by coexisting α thalassaemia, G6PD deficiency and South-East Asian ovalocytosis; in populations where all three are common the sensitivity may be as low as 70% [265].

Suitable methods for the quantification of haemoglobin A₂ include haemoglobin electrophoresis followed by elution and spectrophotometric estimation (only suitable when a laboratory is dealing with small numbers of samples and other techniques are not available), microcolumn chromatography, capillary electrophoresis and HPLC. Quantification of haemoglobin A₂ by scanning densitometry (Fig. 3.38) is not sufficiently precise to be used in the diagnosis of β thalassaemia trait and IEF has not been validated for this purpose.

The percentage of haemoglobin A₂ is dependent on the precise mutation present. In most cases of heterozygosity for β^0 or severe β^+ thalassaemia the haemoglobin A₂ is between 4% and

5% whereas when there is heterozygosity for mild β^+ thalassaemia there is usually 3.6–4.2% of haemoglobin A₂ [266]. Higher percentages are seen in β thalassaemia trait consequent on deletion of the 5' part of the β globin gene (see Table 3.7). In addition, promoter mutations, such as -88 C→T and -29 A→G, and mutations of the initiation codon, such as initiation codon A→G, are associated with higher levels [266]. By definition, in silent β thalassaemia the haemoglobin A₂ percentage is normal. For example, with a C→G mutation at position 6, 3' to the termination codon the mean level is 2.4% [266]. A normal haemoglobin A₂ can also result from coinheritance of δ thalassaemia.

When haemoglobin F is elevated in β thalassaemia trait it usually comprises between 2% and 7% of total haemoglobin. A further rise occurs in pregnancy, paralleling that which occurs in haematologically normal pregnant women [224]. Elevation is usual when β

Table 3.8 Some inherited causes of an increased percentage of haemoglobin A₂ [244–258].**Disorders of globin genes**

β thalassaemia trait (almost all cases)

Haemoglobin E trait [244]

Vietnamese/South-East Asian type of deletional hereditary persistence of fetal haemoglobin (which spares the δ gene)

Hereditary high haemoglobin A₂ with autosomal dominant inheritance [245]Hereditary high haemoglobin A₂ resulting from a mutation in the promoter of the δ gene [246] or a haemoglobin A₂ variant [247]*KLF1* variants* (also have InLu phenotype and haemoglobin F varying from normal up to 4.1 [229] or 4.4 [249] or 4.7% [250])Triple α reported to cause a mild elevation [248] but not confirmed [251] and in a third study this was rare (2 cases with haemoglobin A₂ of only 3.5 and 3.6%) [249]

Unstable haemoglobin

Sickle cell trait†

Sickle cell anaemia, particularly if there is coexisting α thalassaemia†

Sickle cell/β⁰ thalassaemia†

Heterozygosity for certain other β chain variants, e.g. haemoglobin Leslie [252]

G^Aγ fusion (one family) [253]

Haemoglobin anti-Lepore Hong Kong (17–19%) [254]

Disorders unrelated to globin genes

Congenital dyserythropoietic anaemia (some cases among Israeli Bedouins) [255]

Hereditary spherocytosis during acute haemolysis (one case, associated increase in haemoglobin F) [256]

Down syndrome infants (up to 80 days of age) [257]

Pseudoxanthoma elasticum [258]

* Mild increase, e.g. 3.3–4.4%.

† But note that if haemoglobin A₂ is measured by HPLC there is also a factitious rise as post-translationally modified haemoglobin S has the same retention time as haemoglobin A₂.

thalassaemia trait is caused by deletion of the 5' part of the β globin gene. The level of haemoglobin F is influenced not only by the nature of the mutation causing the β thalassaemia but also by coinheritance of non-deletional HPFH variants, some of which are quite common. A Kleihauer test shows that distribution is heterogeneous. Quantification of haemoglobin F is not essential for diagnosis of β thalassaemia, which is dependent on detection of an increased concentration of haemoglobin A₂. However, if a patient has red cell indices suggestive of thalassaemia but has a normal haemoglobin A₂ percentage, it is essential to exclude elevation of haemoglobin F since δβ thalassaemia is an alternative diagnosis. Quantification of haemoglobin F will be automatically available if haemoglobin A₂ is quantified by HPLC. If cellulose acetate electrophoresis and microcolumn chromatography are employed as the routine diagnostic techniques the electrophoretic strip should be carefully inspected for a prominent haemoglobin F band. A concentration above 2% can usually be detected visually and it is then possible to select samples for quantification, e.g. by alkali denaturation. Similarly, the presence of haemoglobin Lepore should be excluded by cellulose acetate electrophoresis, IEF, HPLC or capillary electrophoresis. If β and δβ thalassaemia and 'thalassaemic haemoglobinopathies' such as haemoglobins E and Lepore have been excluded then the most likely explanation of 'thalassaemic' red cell indices, in most ethnic groups, is α thalassaemia. γδβ thalassaemia is also a possible explanation but is very rare. Other patients will have iron deficiency anaemia with atypical red cell indices. Children, in particular, may have iron deficiency anaemia with an elevated red cell count. In adults with both polycythaemia vera and iron deficiency the red cell indices are very similar to those of thalassaemia trait, although the RDW tends to be higher.

A low serum ferritin indicates that there is iron deficiency but does not exclude a diagnosis of β thalassaemia trait. An increased red cell protoporphyrin, which has been used as a screening test for iron deficiency, has been found to be elevated in 51% of cases of β thalassaemia

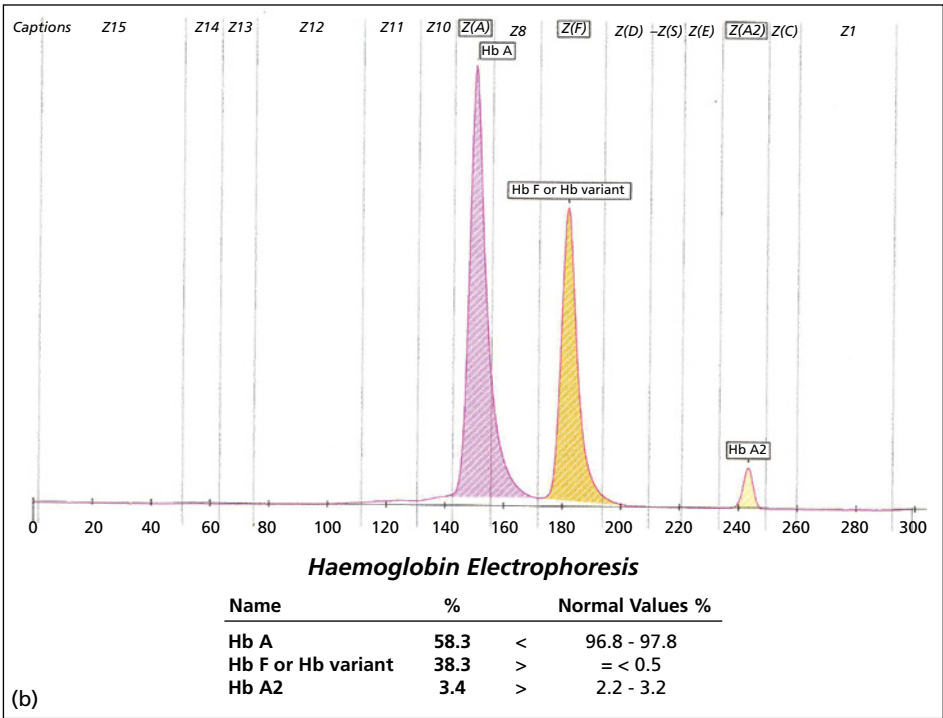
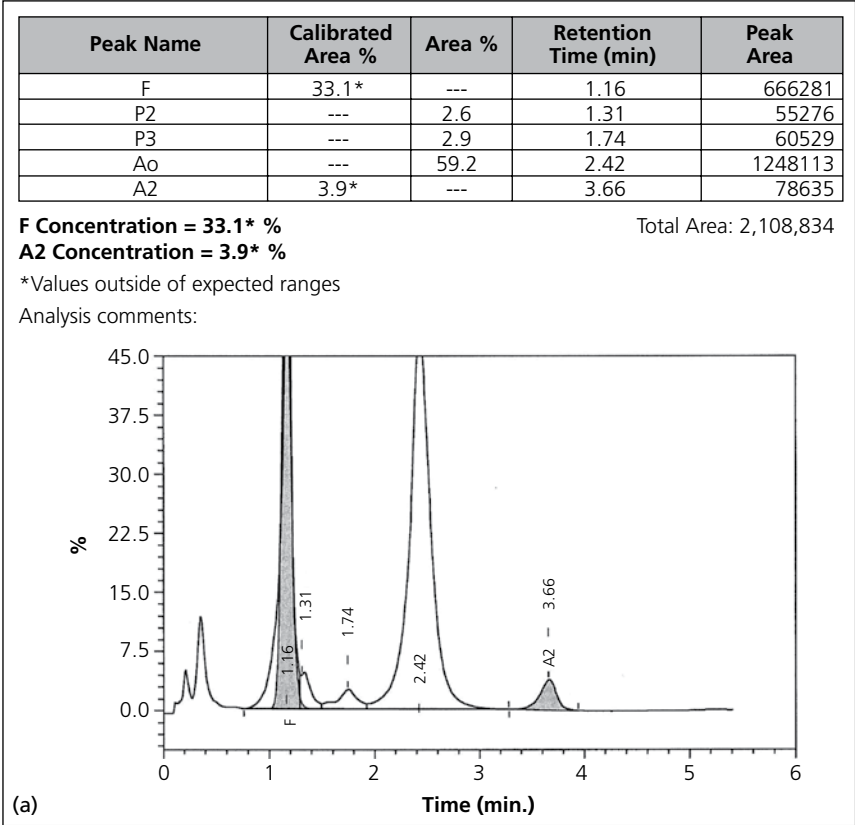


Fig. 3.36 Increased haemoglobin A₂ in a three-month-old baby with β thalassaemia trait: (a) HPLC (Bio-Rad variant II); and (b) capillary electrophoresis (Sebia Capillarys) (same patient as Fig. 3.28).

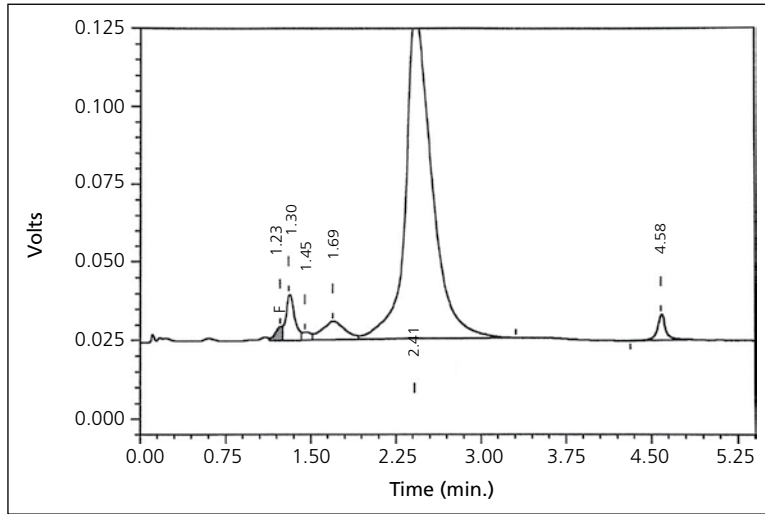


Fig. 3.37 HPLC chromatogram (Bio-Rad Variant II) in a patient with homozygosity for haemoglobin A_2' ; normal haemoglobin A_2 is absent, indicating the patient has no normal δ gene; the A_2' was 2.2% and was in the 'S window' with a retention time of 4.58 minutes; from left to right, the peaks are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobin A_0 and haemoglobin A_2' .

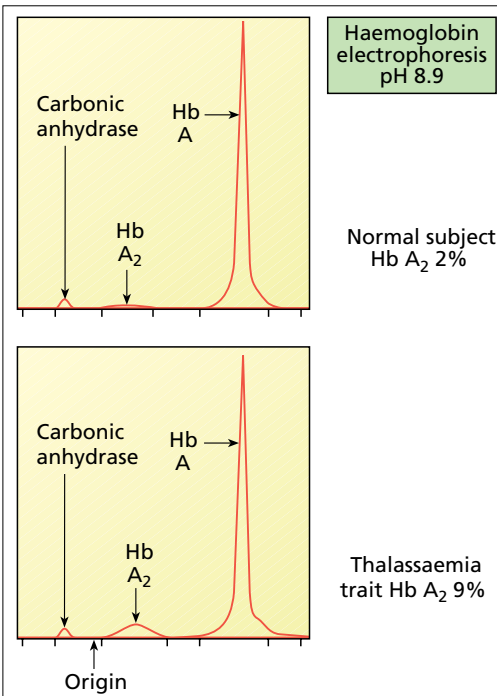


Fig. 3.38 Densitometric scan of an electrophoretic strip in β thalassaemia trait and a haematologically normal subject showing increased haemoglobin A_2 in β thalassaemia trait. This technique is not sufficiently precise to be used for reliable diagnosis but is shown here for illustrative purposes.

trait [88]. This test is therefore of limited value in distinguishing between these two conditions.

It should be noted that β thalassaemia trait can be caused by heterozygous inactivating mutations of *KLF1*, which can cause microcytosis (MCV down to 68 fl and MCH down to 21 pg), increased haemoglobin A_2 (up to 4.9%) and increased haemoglobin F (up to 7.4%) [250]. When a *KLF1* mutation is coinherited with β^0 thalassaemia, there can be a severe deficiency of haemoglobin A synthesis *in utero* and at birth but with improvement predicted to occur thereafter [267]. Homozygosity for a *KLF1* mutation can lead to β thalassaemia intermedia [268].

Problems in the diagnosis of β thalassaemia trait

Neonatal period. β thalassaemia heterozygosity cannot be diagnosed from the haemoglobin A_2 percentage in neonates as levels are low. However, by 6–12 months of age the average haemoglobin A_2 percentage is higher than in other infants [269, 270] and by six months of age the A_2 percentage does not overlap with values seen in babies with normal globin genes (Table 3.9) [270]. It has been noted that in the neonatal period the haemoglobin A percentage

Table 3.9 Haemoglobin A₂ percentage in normal babies and babies with β thalassaemia heterozygosity (adapted from [270]); the mean and 95% ranges are shown.

	Normal babies		Babies with β thalassaemia heterozygosity	
	Number	Mean (95% range) (%)	Number	Mean (95% range) (%)
Birth	16	0.4 (0–0.8)	31	0.5 (0.1–0.9)
3 months	8	1.7 (1.1–2.3)	12	3.2 (1.8–4.6)
6 months	8	2.5 (1.9–3.1)	10	4.8 (3.4–6.2)
9–10 months	6	2.5 (1.7–3.3)	14	5.1 (4.1–6.1)
1 year	5	2.5 (1.9–3.1)	8	4.8 (4.0–5.6)

is usually less than 15% [271]. The rate of decline in haemoglobin F is slower than in haematologically normal infants and adult levels are not reached until well into childhood [224].

Silent and almost silent β thalassaemia. There are β gene variants that cause minor or no haematological abnormalities in heterozygotes but that nevertheless can cause clinically significant disease in homozygotes and compound heterozygotes. It is convenient to divide these variants into two groups designated 'silent β thalassaemia trait' and 'almost silent β thalassaemia trait'. In silent β thalassaemia trait both the red cell indices and the haemoglobin A₂ percentage are normal. It is inevitable that most of these cases will be missed in the routine diagnostic and screening laboratories. In almost silent β thalassaemia trait, red cell indices are abnormal but the haemoglobin A₂ percentage is not increased. The haematological phenotype thus resembles that of α thalassaemia trait. Both silent and almost silent β thalassaemia trait can be detected by studies of rates of globin chain synthesis and DNA analysis. Silent and almost silent β thalassaemia trait can also be referred to as 'normal A₂ β thalassaemia' although it should be noted that there are other causes of β thalassaemia trait with a normal haemoglobin A₂ concentration. Some mutations are so mild that they are always silent, e.g. the +1480 C→G mutation in Greek populations [266, 272]. In other mutations with a greater reduction in β chain synthesis the phenotype in heterozygotes

varies from silent or almost silent to mild β thalassaemia trait. For example, fewer than half of heterozygotes for –101 C→T are truly silent [272]. In one study of 45 heterozygotes, 17 subjects (38%) were completely silent, the same number had a normal MCH but an elevated haemoglobin A₂, four (9%) had an abnormal MCH but a normal A₂ and in four subjects (9%) both the MCH and the haemoglobin A₂ were abnormal [273]. The others had only a modest elevation of haemoglobin F. Among individuals identified only because of a child with β thalassaemia intermedia, half were completely silent, suggesting ascertainment bias in the larger group [273].

Some of the variants that may lead to a silent or almost silent β thalassaemia phenotype, and the ethnic groups in which they occur, are shown in Table 3.10 [9, 182, 186, 193, 266, 272–288]. The most common mutations responsible for silent β thalassaemia are –101 C→T and –92 C→T. Mean values reported for individuals carrying the latter mutation are MCV 83.9 fl, MCH 28.6 pg and haemoglobin A₂ 3.4% [280]. Almost silent β thalassaemia trait results from a small group of mild β thalassaemia mutations, such as CAP +1 A→C in South Asian (Indian) populations and, occasionally from IVS1 6 T→C in Mediterranean individuals. Heterozygotes for the CAP +1 mutation have, for example, been observed to have the following mean values: MCV 79 fl, MCH 24.7 pg, Hb A₂ 3.4% (data kindly provided by Dr John Old). Heterozygotes for IVS1 6 T→C had an MCV ranging from 60 to 78 fl and an A₂ percentage ranging from 3.2% to

Table 3.10 Causes of normal haemoglobin A₂-β-thalassaemia [9, 182, 186, 193, 266, 272–288].

Mutation	Origin	Usual haemoglobin A ₂ (mean or range) %	Usual MCH (mean or range) pg	Usual MCV (mean or range) fl
Silent β thalassaemia trait (normal MCV, MCH and haemoglobin A₂ percentage)				
–101 C→T	Mediterranean	3.3	28	85
–92 C→T	Mediterranean	3.5	28	82
CAP + 8 C→T [186]	Chinese	3.0	33.7	98
CAP + 10(–T) [186, 274]	Greek	2.5–2.7	30–33	94–102
IVS2 844 C→G	Mediterranean (Italian)	3.5	28–29	85
CAP +33 C→G [275]	Mediterranean (Greek Cypriot)	3.0	29	86
CAP +1480 C→G (termination codon +6 C→G) [186, 266, 272]	Mediterranean (Greek)	1.9–3.4	20–31	79–95
Almost silent β thalassaemia trait (reduced MCV and MCH, normal haemoglobin A₂ percentage)				
IVS1 6 T→C	Mediterranean*	3.5	23	71
Codon 27G→T) (haemoglobin Knossos†)	Mediterranean and Middle Eastern	2.1	25	71
IVS1 5 G→A Corfu δβ‡	Mediterranean			
IVS1 128 T→G	Saudi	3.5	25	70
CAP +1 A→C	South Asian	3.4	25	80
Mutation not linked to β globin gene cluster [193]	Italian	1.6 [§]	23.5 [§]	76 [§]
CAP +22 G→A [270]	Turkish, Bulgarian	3.9	23.5	79
Poly A T→C	African			
Other β thalassaemia mutations with a defective δ gene in <i>cis</i> or in <i>trans</i>	Mediterranean including Sardinia			
Indices typical of thalassaemia trait but haemoglobin A₂ percentage normal				
β thalassaemia caused by deletion of the locus control region	Various	Normal	Typical of β thalassaemia	typical of β thalassaemia
γδβ thalassaemia	Various	Normal	Typical of β thalassaemia	typical of β thalassaemia

MCH, mean cell haemoglobin; MCV, mean cell volume.

Dr John Old from the National Haemoglobinopathy Reference Laboratory, Oxford, kindly provided some of the data on which this table is based. Further information from references [9, 182, 186, 193, 266, 272, 275, 278].

* Sometimes referred to as the Portuguese mutation but is common throughout the Mediterranean area. Most common mutation in Malta where half of heterozygotes for this mutation were demonstrated to have a haemoglobin A₂ percentage below that usually used for diagnosis of β thalassaemia heterozygosity [277].

† Does not separate from haemoglobin A on alkaline or acid electrophoresis or HPLC but separates on isoelectric focusing; 35–40% of haemoglobin; often has a δ⁰ thalassaemia mutation in *cis* and haemoglobin A₂ is then normal in heterozygotes and absent in homozygotes [279].

‡ Actually represent δ⁰ with β⁺ thalassaemia in *cis*.

§ One case reported; compound heterozygotes with this mutation and β⁰ thalassaemia have thalassaemia intermedia or major.

4.6% [277]. Individuals carrying these mutations will sometimes have the phenotype of mild β thalassaemia trait and sometimes of almost silent thalassaemia trait.

Other causes of β thalassaemia trait with a normal haemoglobin A_2 percentage. Both inherited and acquired abnormalities can lead to β thalassaemia trait with a normal haemoglobin A_2 concentration.

Iron deficiency has been found to lower the haemoglobin A_2 percentage both in individuals with no defect of globin genes and in those with thalassaemia trait [281–286], although not all studies have confirmed this observation [287]. In one study of four patients the mean haemoglobin A_2 percentage increased from 3.07 to 3.81 when iron deficiency was treated [286]. This is not likely to lead to any diagnostic problem in individuals with a genetic abnormality that is usually associated with a marked elevation of haemoglobin A_2 . However, in those with mild β thalassaemia mutations, in which the haemoglobin A_2 is usually only slightly elevated, results may fall to within the normal range. For these reasons it is preferable not to attempt to exclude a diagnosis of β thalassaemia trait in patients with severe or moderately severe iron deficiency but rather to correct the iron deficiency and then measure A_2 percentage if the red cell indices do not return to normal. An exception to this generalisation is in antenatal screening of pregnant women where diagnosis of β thalassaemia trait is required without delay. In such circumstances it may be necessary to screen the partner for haemoglobinopathies, and if he is found to have a variant which could interact with β thalassaemia trait to produce a clinically significant condition, consider proceeding to molecular analysis in women with borderline haemoglobin A_2 percentages. Folic acid deficiency can also lower the percentage of haemoglobin A_2 and interfere with the diagnosis of β thalassaemia trait although, in individuals who do not have β thalassaemia trait, megaloblastic anaemia – resulting from deficiency of either vitamin B_{12} or folic acid – causes a significant rise in

haemoglobin A_2 percentage, proportional to the degree of anaemia [282].

The phenotype of abnormal red cell indices with a normal haemoglobin A_2 concentration can also result from the coinheritance of δ thalassaemia (in *cis* or *trans*) and a β^+ or β^0 thalassaemia mutation that would normally lead to a high haemoglobin A_2 . Such coinheritance of β and δ thalassaemia trait occurs particularly in Sardinians and in Cypriots and has also been reported in China. The prevalence of δ^0 thalassaemia in Sardinia is more than 1% [288]. The so-called ‘Sardinian $\delta\beta$ thalassaemia’ (see page 171) actually represents coinheritance of β^0 thalassaemia and non-deletional HPFH resulting from a mutation in the *HBB1* gene; haemoglobin A_2 averages around 2.5%. Haemoglobin Knossus, a ‘thalassaemic haemoglobinopathy’, is also responsible for some cases of β thalassaemia trait with a normal (or only slightly elevated) haemoglobin A_2 . This is caused by a δ^0 thalassaemia gene in *cis*; this variant haemoglobin is not detectable by haemoglobin electrophoresis under standard conditions [182]. Homozygosity for δ^0 thalassaemia can lead to β thalassaemia heterozygosity with absent haemoglobin A_2 [289].

It should also be noted that $\epsilon\gamma\delta\beta$ thalassaemia and deletions leading to loss of the upstream locus control region will not have any elevation of haemoglobin A_2 , and will be missed by many antenatal screening programmes, but have a similar significance to β thalassaemia heterozygosity.

Coinheritance of α and β thalassaemia

When β thalassaemia is coinherited with heterozygosity for α^0 thalassaemia or either heterozygosity or homozygosity for α^+ thalassaemia the MCV and MCH are higher and the haemoglobin A_2 percentage is lower; the haemoglobin A_2 may be normal [290]. The coinheritance of either heterozygous α^0 thalassaemia or homozygous α^+ thalassaemia trait and mild β thalassaemia (e.g. mutations such as CAP +1 A→C trait) makes it more likely that the diagnosis of β thalassaemia trait will be missed. In a small proportion of such individuals the MCV

and MCH are normal. The coinheritance of α thalassaemia trait can lead to a mutation that would otherwise have presented with a normal haemoglobin A_2 but abnormal red cell indices having normal findings with regard to both red cell indices and haemoglobin A_2 concentration (i.e. an almost silent thalassaemia becomes a silent thalassaemia).

When an individual has both haemoglobin H disease and β thalassaemia heterozygosity, the haemoglobin A_2 can be normal. Seven patients reported by Liang et al. [291] had haemoglobin A_2 varying from 2.8% to 3.9% with a mean value of 3.5%. Of another series of seven patients, haemoglobin A_2 percentage ranged from 2.1 to 4.8 with two values clearly being within the normal range [160].

Coinheritance of β thalassaemia and either δ thalassaemia or a δ chain variant

The coinheritance of β and δ thalassaemia can lead to a normal haemoglobin A_2 percentage or, if there is homozygosity for δ thalassaemia, absent haemoglobin A_2 . The red cell indices remain typical of thalassaemia trait and the diagnosis can be made by DNA analysis.

A diagnostic problem can also occur when an A_2 variant is present. Failure to detect a split A_2 band or peak, indicating either an α or a δ chain variant, can cause the diagnosis of β thalassaemia trait to be missed as a result of an incorrect estimation of total haemoglobin A_2 percentage.

The significance of borderline haemoglobin A_2

A borderline high haemoglobin A_2 (e.g. 3.4–3.9%) or even a high normal haemoglobin A_2 (e.g. 3.0–3.5%) can create diagnostic problems. Giambona et al. investigated 23485 Sicilian subjects with haemoglobin A_2 of 3.4–3.9% and normal or abnormal red cell indices, of whom 410 had had molecular analysis. β heterozygosity was found in 15%, sometimes with coexisting δ thalassaemia, of 315 Italian individuals [292]. Galanello et al. had previously investigated 125 individuals of Sardinian descent with haemoglobin A_2 between 3.0% and

3.5% (with normal or abnormal red cell indices) and found 33 individuals (26%) to have β thalassaemia heterozygosity (either a mutation typically associated with mild β thalassaemia or coinheritance of δ and β thalassaemia) [293]. Both Sicily and Sardinia are high prevalence areas for δ and β thalassaemia. The proportion of patients with a borderline haemoglobin A_2 having a significant genetic abnormality would obviously be lower in a low prevalence area.

β thalassaemia trait with normal red cell indices but elevated haemoglobin A_2

There are occasional patients with heterozygosity for mild β thalassaemia variants who have an elevated haemoglobin A_2 percentage despite normal red cell indices. This was found, for example, in 17 of 45 individuals with heterozygosity for $-101\text{ C}\rightarrow\text{T}$ [273].

In addition, coinheritance of α and β thalassaemia can lead to normal red cell indices and more balanced chain synthesis but with haemoglobin A_2 being elevated [294]. This is seen mainly in those with deletion of two of the four α genes or with non-deletional α thalassaemia. Gasperini et al. [245] found that of 315 Italian individuals with normal red cell indices and a high haemoglobin A_2 , 313 had coexisting α and β thalassaemia.

Acquired abnormalities, such as liver disease and hypothyroidism, can raise the MCV and MCH of patients with β thalassaemia trait into the normal range.

Cases of these types will be detected if estimation of haemoglobin A_2 percentage is carried out regardless of the red cell indices (e.g. in laboratories using HPLC or capillary electrophoresis for screening for haemoglobinopathies and thalassaemias) but will necessarily be missed if A_2 measurement is performed only on individuals with abnormal indices.

Coinheritance with other abnormalities of globin chain synthesis

Coinheritance of haemoglobin H disease and β thalassaemia has been discussed earlier.

Although the condition may be milder than typical haemoglobin H disease, abnormalities are more marked than in typical β thalassaemia trait, with significant anaemia, more marked microcytosis, reduced MCHC, increased RDW and significant iron overload.

Coinheritance with three rather than two α globin genes leads to worse chain imbalance with a variable phenotype. Some patients have anaemia and microcytosis whereas others have a more severe thalassaemia intermedia phenotype. Occasionally this can also cause a significant haemolytic anaemia with a normal MCV but a low MCH [295]. Coinheritance of β thalassaemia trait and quadruple α can lead to thalassaemia major [296].

Coinheritance of β thalassaemia trait and non-deletional HPFH leads to a modification of the phenotype of β thalassaemia [297]. The MCH and MCV are higher and the haemoglobin A₂ percentage is somewhat lower; the serum soluble transferrin receptor concentration is lower.

There are several variant haemoglobins that, when coinherited with β thalassaemia trait, give the clinical picture of β thalassaemia intermedia (see Table 3.11), although the vast majority do not; the latter include, for example, haemoglobin J-Sardegna and haemoglobin Norfolk, which show no interaction [298]. The inheritance of a β thalassaemia allele together with a high affinity haemoglobin, such as haemoglobin Crete or haemoglobin San Diego, does not prevent the development of polycythaemia [298].

Coinheritance with other erythrocyte abnormalities

Coinheritance of β thalassaemia and hereditary elliptocytosis may cause a symptomatic haemolytic anaemia necessitating splenectomy [299]. Coinheritance with South-East Asian ovalocytosis alters the blood film features, raises the MCHC and normalises the osmotic fragility but does not alter the clinical severity [300].

Dominant β thalassaemia

Most individuals who are heterozygous for a β thalassaemia mutation have clinicopathological features described as 'thalassaemia minor' (the blood count and film are abnormal but there are no abnormal physical findings or symptoms). However, some mutations produce clinically apparent abnormalities in the heterozygous state, including splenomegaly, anaemia, jaundice and an increased incidence of gallstones. This is referred to as dominant β thalassaemia. Dominant β thalassaemias are rare but cases are found scattered throughout the world. The clinicopathological features are those of thalassaemia intermedia with both ineffective haemopoiesis and a haemolytic component. Occasionally, as with haemoglobin Boston-Kuwait [301] or with a highly unstable elongated β chain due to an intragenic duplication [302], the phenotype is that of thalassaemia major. Red cell survival is shorter than in typical β thalassaemia trait and the reticulocyte count is increased. Patients may require occasional blood transfusions. There is extramedullary haemopoiesis and iron overload can occur. The blood film (Fig. 3.39) is usually very abnormal with prominent basophilic stippling and circulating nucleated red cells. The bone marrow shows erythroid hyperplasia and dyserythropoiesis (Fig. 3.40). Red cell inclusions are detected on incubation with vital dyes. This gave rise to an earlier designation, 'inclusion body β thalassaemia', but this is a less appropriate term than 'dominant β thalassaemia' since cases of thalassaemia major also have erythroblast inclusions. Erythroblast inclusions are composed of excess α chains and abnormal β chains (Fig. 3.41) whereas in β thalassaemia major they are composed of excess α chains alone.

More than 40 dominantly inherited alleles have now been described [182, 188, 302]. In comparison with the common recessive forms of β thalassaemia, dominantly inherited β thalassaemia much more often results from mutations in the 3' third of exon 2 or in exon 3 rather than in exon 1 or the 5' part of exon 2

Table 3.11 Genotypes that can produce the phenotype of β thalassaemia intermedia [177, 182, 188, 190, 250, 252, 254, 279, 298, 303, 308, 310, 311, 324–337].

With two β thalassaemia alleles

Homozygosity or compound heterozygosity for mild or very mild β^+ thalassaemia alleles, e.g. CAP+1 A \rightarrow C, IVS1 6 T \rightarrow C, +33 C \rightarrow G, -101 C \rightarrow T, -88 C \rightarrow T, -87 C \rightarrow G, -29 A \rightarrow G* and some polyadenylation signal mutations, particularly if coinherited with α thalassaemia trait (α^0 thalassaemia trait or homozygous α^+ thalassaemia trait)

Compound heterozygosity for a mild or very mild β^+ thalassaemia allele and a severe β^+ or β^0 thalassaemia allele, particularly when ameliorated by coinheritance of α thalassaemia trait ($-\alpha/-\alpha$ or $--/\alpha\alpha$) or non-deletional HPFH (either -158 $^c\gamma$ C \rightarrow T mutation, $^c\gamma$ or $^{\wedge}\gamma$ promoter mutations or enhanced synthesis of γ chain not linked to the β globin locus†)

Homozygosity or compound heterozygosity for β^+ thalassaemia if ameliorated by α^0 thalassaemia heterozygosity or α^+ thalassaemia homozygosity or non-deletional α thalassaemia or the genotype of haemoglobin H disease‡; compound heterozygosity for β^+ and β^0 thalassaemia if ameliorated by the genotype of haemoglobin H disease

Homozygosity or compound heterozygosity for severe β^+ or β^0 alleles when ameliorated by coinheritance of non-deletional HPFH, particularly if homozygous (e.g., either -158 $^c\gamma$ C \rightarrow T mutation, -196 $^{\wedge}\gamma$ C \rightarrow T or enhanced synthesis of γ chain not linked to the β globin locus‡) or a heterozygous inactivating mutation of *KLF1* [250] or α thalassaemia (deletion of 2 or 3 α genes or non-deletional α thalassaemia)

Homozygosity for β^0 thalassaemia caused by 5' deletions or point mutations of the β promoter leading to enhanced haemoglobin F and sometimes haemoglobin A₂ synthesis

Homozygosity for Spanish $\delta\beta$ thalassaemia

Homozygosity for 'Corfu $\delta\beta$ thalassaemia' (coinheritance of δ and β thalassaemia with a β gene mutation in *cis* that down regulates the gene and is associated with increased haemoglobin F synthesis) [188, 334]§

Compound heterozygosity for $\delta\beta$ and β^+ or β^0 thalassaemia or homozygosity for $\delta\beta$ thalassaemia

Homozygosity for haemoglobin Lepore

Homozygosity for haemoglobin Knossos [279]

Homozygosity or compound heterozygosity for β^0 or β^+ thalassaemia with no detectable ameliorating factors

Homozygosity for haemoglobin Malay or compound heterozygosity for haemoglobin Malay and haemoglobin E [177]

With one β thalassaemia allele and a variant haemoglobin or HPFH or transcription factor gene mutation

Compound heterozygosity for haemoglobin E or Hb Knossos and β thalassaemia or haemoglobin Lepore

Compound heterozygosity for β^0 thalassaemia and haemoglobin D-Punjab, haemoglobin C, haemoglobin O-Arab, haemoglobin City of Hope, haemoglobin Siriraj, haemoglobin Beograd or the unstable haemoglobins, haemoglobin Acharnes, haemoglobin Arta or haemoglobin Lulu Island [298, 308, 324, 326]

Compound heterozygosity for β or $\delta\beta$ thalassaemia and deletional HPFH

Compound heterozygosity for β thalassaemia and haemoglobin anti-Lepore Hong Kong [254]

Compound heterozygosity for β thalassaemia and haemoglobin Zurich [327]

Coinheritance of β thalassaemia and *SUPT5H* mutation [190]

With one β thalassaemia allele

β^+ thalassaemia or β^0 thalassaemia co-inherited with heterozygosity or homozygosity for triple α or quadruple α (total of 5–7 α genes), e.g. $\alpha\alpha/\alpha\alpha\alpha$, $\alpha\alpha\alpha/\alpha\alpha\alpha$, $-\alpha^{37}/\alpha\alpha\alpha\alpha$, $\alpha\alpha/\alpha\alpha\alpha\alpha$, $\alpha\alpha\alpha\alpha/\alpha\alpha\alpha\alpha$ [328, 329] or duplication of entire α globin cluster $\alpha\alpha/\alpha\alpha.\alpha\alpha$ [305] sometimes with triple α coinherited $\alpha\alpha\alpha/\alpha\alpha.\alpha\alpha$ [311]

Dominant β thalassaemia due to very unstable β globin chain [303] due to an initiator codon mutation [310]

Somatic deletion of one β globin locus leading to mosaicism [332, 333]

HPFH, hereditary persistence of fetal haemoglobin.

* In black populations, because the same chromosome carries -158 $^c\gamma$ C \rightarrow T; in Chinese populations is associated with thalassaemia major [182].

† However, the genotype of haemoglobin H disease aggravates $\beta^0\beta^0$ thalassaemia.

‡ For example, Xp22.2-linked, 6q23-linked or 8q11-linked.

§ 88–90% haemoglobin F and mild anaemia as γ genes are upregulated [334].

Fig. 3.39 Blood film in dominant β thalassaemia showing anisocytosis, poikilocytosis and basophilic stippling. MGG $\times 100$. (With thanks to Dr Ayed Eden.)

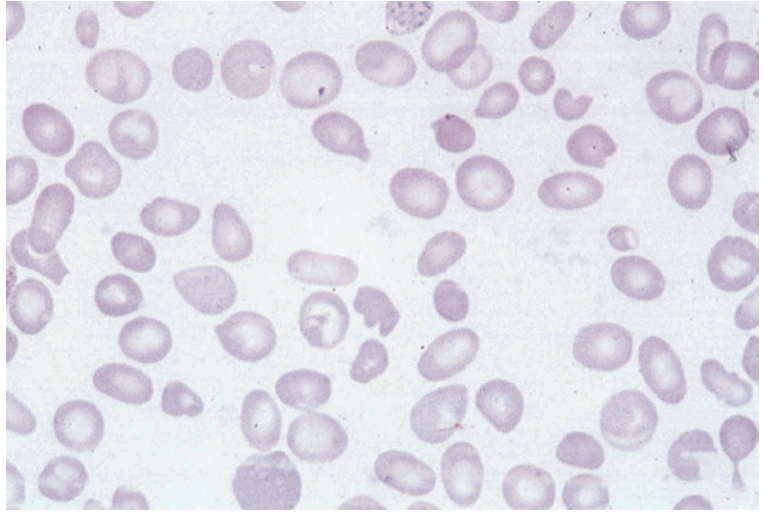


Fig. 3.40 Bone marrow aspirate in dominant β thalassaemia showing erythroid hyperplasia and dysplasia. MGG $\times 100$. (With thanks to Dr Ayed Eden.)

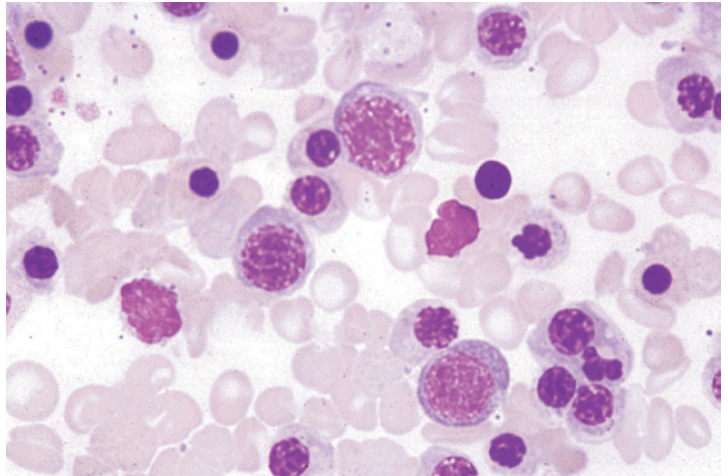
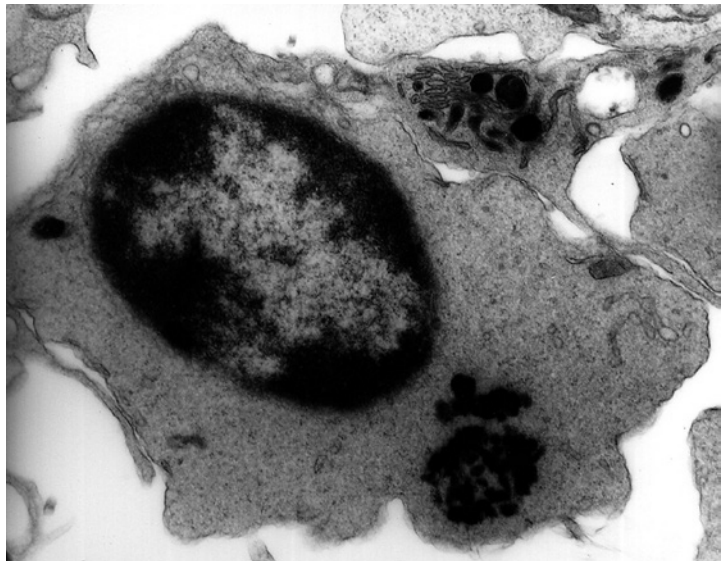


Fig. 3.41 Ultrastructural examination in dominant β thalassaemia. (By courtesy of the late Professor Sunitha N. Wickramasinghe.)



and is associated with substantial amount of mutant mRNA [182, 303]. Mutations responsible typically lead to production of either a truncated or elongated β globin chain that is very unstable and may coprecipitate with normal α chains [304, 305]. These mutations have a dominant negative effect consequent on the presence of this abnormal protein, in comparison with the haploinsufficiency recessive effect of other mutations. Haemopoiesis is not only ineffective but also dysplastic. Molecular mechanisms include the following [182, 188, 303, 305–309]:

- nonsense mutations in the third exon leading to a truncated unstable β chain;
- mis-sense mutations, particularly in the third exon but occasionally in the first or second exon;
- frameshift mutations (e.g. small deletions or, less often, insertions or a combination of deletion and insertion) in the third exon, or a mutation resulting in aberrant splicing, leading to a truncated or elongated β chain;
- deletion or insertion of complete codons in the second or third exons leading to destabilisation;
- deletion within the 5' consensus splicing region of the second intron leading to aberrant splicing.

One possible explanation of the particular association between dominant β thalassaemia and exon 3 mutations is that the abnormal globin chain that is synthesised is sufficiently long to bind haem (binding sites being mainly encoded by exon 2) but lacks the residues necessary for $\alpha\beta$ dimer formation (encoded by exon 3) [303]; the aberrant chains are more slowly degraded than the shorter globin chains encoded by more truncated genes. There is therefore damage to red cell precursors. Some of the abnormal β chains produced when there is deletion or insertion of an entire codon may likewise be unable to form $\alpha\beta$ dimers [303].

Many dominant β thalassaemias have been designated 'haemoglobin variants' although it is rare to be able to detect the variant haemoglobin predicted from the DNA sequence. These can also be regarded as hyperunstable haemoglobins [308].

Mutations of the initiator codon can also lead to an unusually severe phenotypic abnormality in heterozygotes with significant anaemia and splenomegaly [310].

Haemoglobin Lepore trait

Unequal crossover during meiosis with deletion of the 3' part of the δ gene and the 5' part of the β gene leads to formation of a $\delta\beta$ fusion gene. The fusion gene encodes a variant $\delta\beta$ fusion chain that is synthesised at a much reduced rate in comparison with normal β chain. The variant haemoglobin produced is designated haemoglobin Lepore (from the family name of the first patient in whom this variant haemoglobin was recognised). Since the extent of the deletion varies, there are several different haemoglobins designated 'haemoglobin Lepore', the most common of which is haemoglobin Lepore Boston/Washington. Others include haemoglobin Lepore Baltimore, haemoglobin Lepore Hollandia, haemoglobin Lepore Leiden [311] and haemoglobin Lepore-ARUP [312]. Haemoglobin Lepore Leiden results from a very complex crossover so that the sequence of gene segments is actually $\delta\beta\delta\beta\delta$ [311]. Haemoglobin Parchman, however, although it is also the result of a complex crossover with a $\delta\beta\delta$ haemoglobin sequence, is without apparent haematological consequences since the β gene is intact [313]; haemoglobin A_2 is reduced. Haemoglobin Palencia is also a $\delta\beta\delta$ hybrid, without clinical consequences and with a normal haemoglobin A_2 , suggesting that the δ gene is retained [314]. Haemoglobin Lepore Boston occurs with a low frequency in a variety of ethnic groups including Italians (particularly from around Naples, and also 0.6% of Sicilians), Greeks (particularly Macedonians), Turks, Spaniards, Balkan populations and individuals with African ancestry (Cubans, Caribbeans, African Americans and African Caribbeans in the UK). Haemoglobin Lepore Baltimore is found in Brazil, Portugal and Italy. Haemoglobin Lepore Hollandia is rare, having been reported in isolated families in Papua New Guinea, Bangladesh and Thailand. Haemoglobin Lepore is a form of β thalassaemia and is important

because of the possibility of interaction with haemoglobin S and with other β thalassaemia alleles. From the functional point of view it can be regarded as a $\delta\beta^+$ thalassaemia.

Laboratory features

The blood count and blood film (Fig. 3.42) features cannot be distinguished from those of β thalassaemia trait.

Haemoglobin electrophoresis shows 5–15% of haemoglobin Lepore with haemoglobin A_2 being reduced, on average, to about half of the normal level. The percentage of haemoglobin Lepore Baltimore in heterozygotes is slightly but significantly higher than the percentage of haemoglobin Lepore Boston [315]. The Haemoglobin A_2 percentage tends to be lower with haemoglobin Lepore Baltimore than with haemoglobin Lepore Boston [315]. Haemoglobin F is sometimes mildly increased; this may be because of linkage to a polymorphism that determines haemoglobin F percentage [315]. At least in Spaniards, the haemoglobin F percentage tends to be higher in association with haemoglobin Lepore Baltimore than in association with haemoglobin Lepore Boston [315]. Haemoglobin Lepore has the same mobility as haemoglobin S on cellulose acetate electrophoresis at alkaline pH (Fig. 3.43) and moves with haemoglobin A at acid pH. On HPLC, it has the same retention time as haemoglobin A_2

(Fig. 3.44). On capillary electrophoresis, the Lepore haemoglobins separate from haemoglobins A, S, E, C and A_2 , appearing in the same zone as haemoglobin D-Punjab and haemoglobin G-Philadelphia (Fig. 3.45). Definitive identification of the exact type of haemoglobin Lepore requires DNA analysis, usually involving GAP-PCR, MLPA or sequencing across the breakpoints.

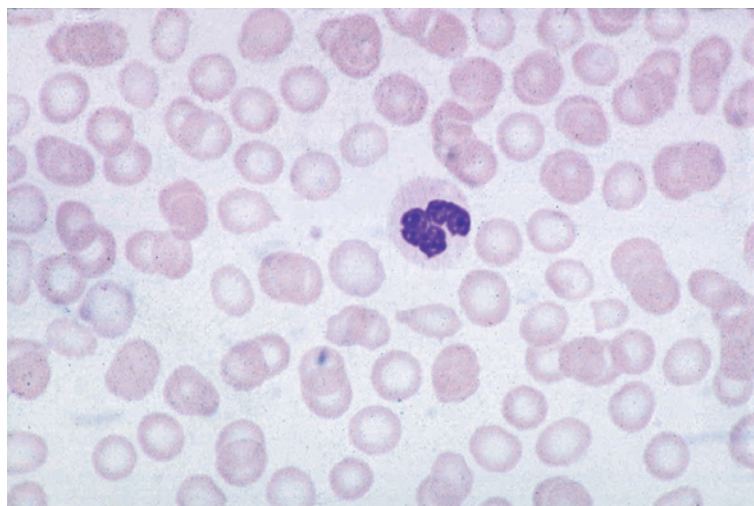
Haemoglobin Lepore homozygosity and compound heterozygosity

Haemoglobin Lepore homozygotes have the clinical and haematological picture of thalassaemia major or thalassaemia intermedia. Haemoglobin electrophoresis shows haemoglobins F and Lepore only. Compound heterozygotes for haemoglobin Lepore and β thalassaemia similarly can have the clinical and haematological features of either thalassaemia major or thalassaemia intermedia. Haemoglobin electrophoresis shows haemoglobins F, Lepore and A_2 with or without some haemoglobin A.

β thalassaemia intermedia/non-transfusion-dependent thalassaemia

β thalassaemia intermedia, also often designated NTDT, refers to a clinical phenotype with diverse genetic explanations. In comparison with a typical patient with β thalassaemia trait,

Fig. 3.42 Blood film in haemoglobin Lepore trait showing hypochromia, microcytosis and mild poikilocytosis; red cell indices were RBC $5.36 \times 10^{12}/l$, Hb 120 g/l, Hct 0.351, MCV 66 fl, MCH 22.2 pg, MCHC 338 g/l. MGG $\times 100$.



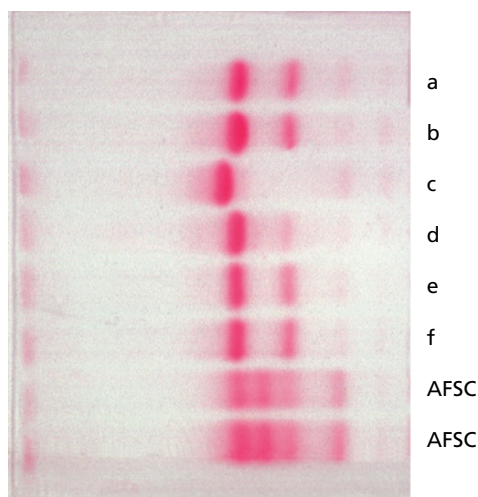


Fig. 3.43 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in haemoglobin Lepore trait (lane d); AFSC indicates a control sample containing haemoglobins A, F, S and C. Haemoglobin Lepore has the same mobility as haemoglobin S.

there are significant clinical problems such as anaemia, splenomegaly, leg ulcers, iron accumulation, extramedullary haematopoiesis, osteopenia and bony deformity. The condition differs from thalassaemia major in that the patient is not dependent on regular blood transfusions for survival, although transfusions may

be needed occasionally (e.g. during intercurrent infection) or may become necessary later in life, although there is no clear point at which severe thalassaemia intermedia becomes thalassaemia major, and the use of regular blood transfusions depends on clinical judgement rather than any particular genetic or laboratory findings. The term neotransfusion-dependent thalassaemia has been suggested when the phenotype changes to transfusion dependency [316].

The severity of β thalassaemia intermedia varies from a condition in which survival without transfusion is barely possible, and there is growth retardation and bony deformity, to a much milder condition that resembles β thalassaemia trait but has a greater degree of anaemia and splenomegaly. There is expansion of the medullary cavity (Fig. 3.46) and there can be extramedullary haemopoiesis. Some patients develop symptoms resulting from pressure on vital organs when extramedullary haemopoietic tissue forms tumour-like masses; these are often in the mediastinum or pleura or within the spinal canal, causing spinal cord compression. In one reported patient extramedullary haemopoietic tissue in the liver formed a tumour-like mass, detectable on computed tomography (CT) scanning (Fig. 3.47) [317]. Iron overload, hypothyroidism and gonadal

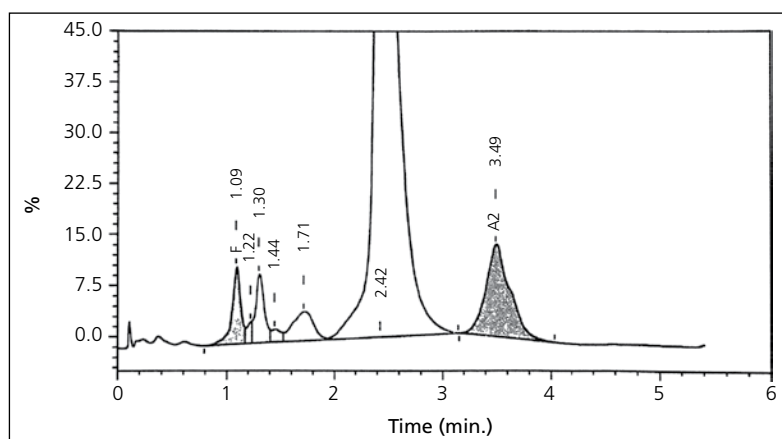


Fig. 3.44 HPLC chromatogram (Bio-Rad Variant II) in haemoglobin Lepore trait; haemoglobin Lepore (plus haemoglobin A_2) was 13.4% and its retention time was 3.49 minutes; from left to right the peaks are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobin A_0 and haemoglobin Lepore plus haemoglobin A_2 (shaded).

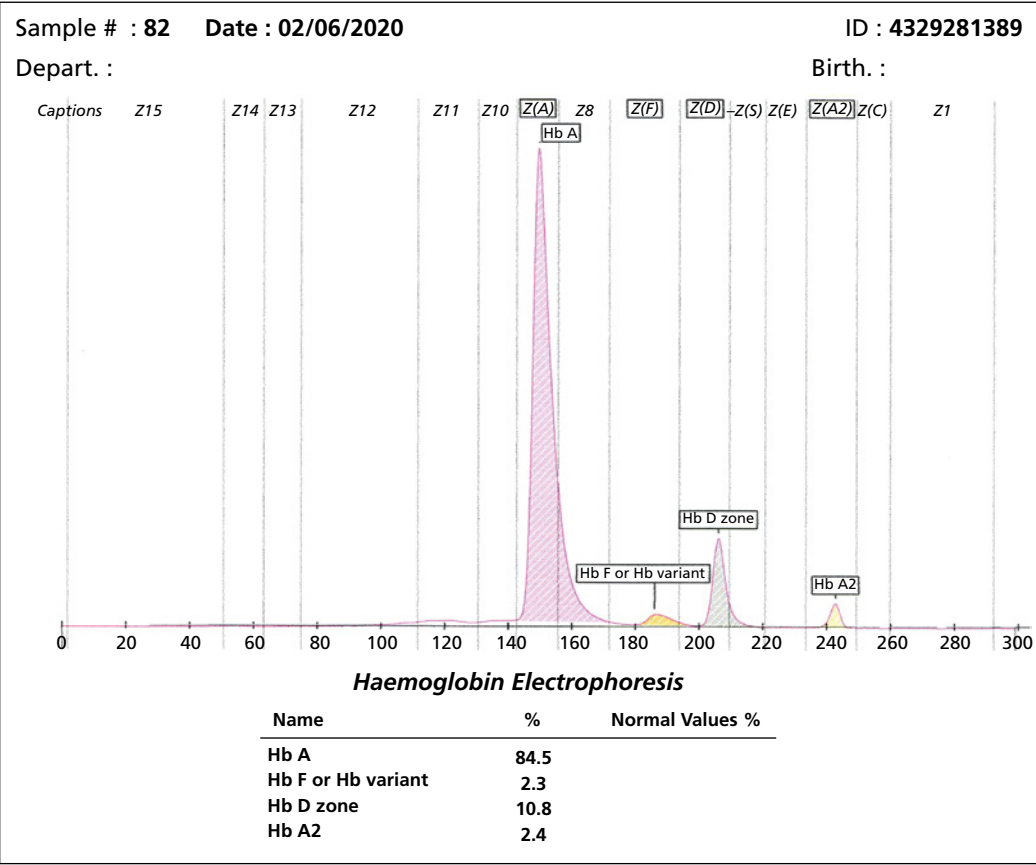


Fig. 3.45 Capillary electrophoresis (Sebia Capillars) in haemoglobin Lepore trait showing the variant haemoglobin in the D zone.

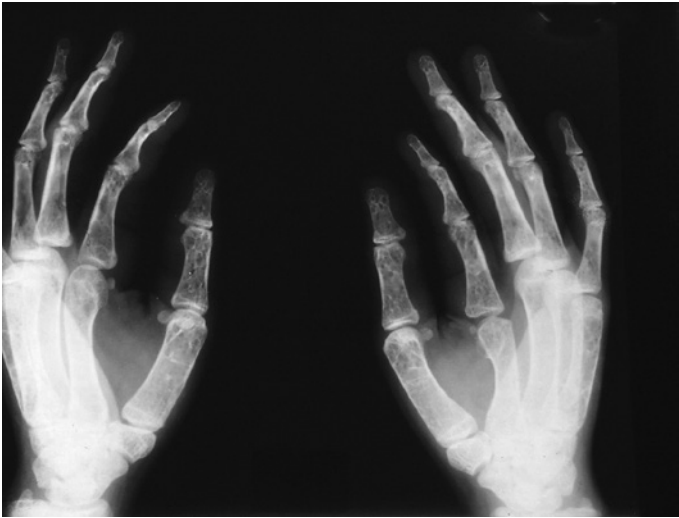


Fig. 3.46 Radiograph of the hands of a patient with β thalassaemia intermedia showing lucent areas that represent expanded erythropoiesis. (With thanks to Dr Saad Abdalla.)

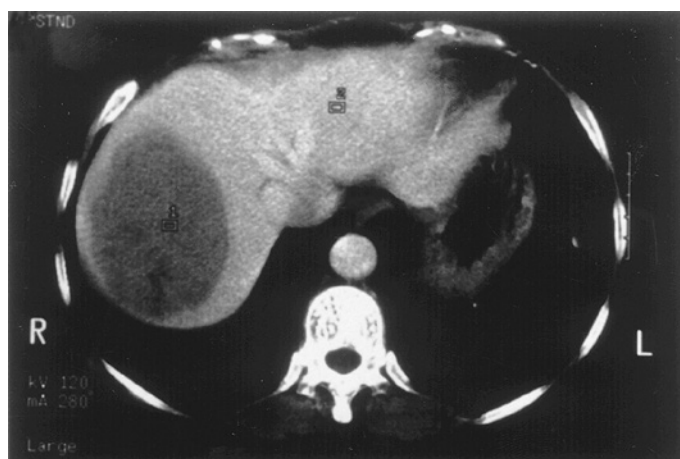


Fig. 3.47 Computed tomography (CT) scan of abdomen showing a tumour-like mass of haemopoietic tissue in the liver in a patient with β thalassaemia intermedia. (With thanks to Dr S.K. Ma and W.Y. Au and by permission of the British Journal of Haematology.)

failure can occur. Hepatic iron is preferentially deposited in hepatocytes rather than macrophages, leading to a disproportionately low serum ferritin. Hepatic complications, in addition to extramedullary haemopoiesis, include fibrosis, cirrhosis and hepatocellular carcinoma resulting from iron overload. The incidence of gallstones is increased. Bone complications include expansion of the medullary cavity, osteoporosis (related in part to gonadal failure) and fractures. Patients with thalassaemia intermedia can develop hypersplenism; splenic sequestration has also been recognised. Cardiovascular complications are common and include congestive cardiac failure, acute pericarditis, chronic pericardial thickening, pulmonary hypertension and incompetence of mitral and aortic valves [318]. There is an increased incidence of venous thromboembolism and portal vein thrombosis, the hypercoagulable state being particularly pronounced after splenectomy and correlating with a lower Hb [319]. Silent cerebral infarction can occur [320]. Pulmonary hypertension, which can lead to right heart failure, is attributable both to recurrent venous thromboembolism and to interstitial fibrosis resulting from iron deposition. Glomerular hyperfiltration is common, with proteinuria occurring in a minority of patients [321]; in small minority of patients, end stage renal failure eventually occurs. Rarely priapism has been described [322]. Cognitive impairment has been reported in adults with β thalassaemia intermedia [323].

The causes of thalassaemia intermedia are summarised in Table 3.11 [177, 182, 188, 190, 250, 252, 254, 279, 298, 303, 308, 310, 311, 324–337]. The condition can occur in patients with either one or two abnormal β genes. Those who are homozygotes or compound heterozygotes for β thalassaemia alleles either have mutations that are usually associated with mild or very mild β^+ thalassaemia or have ameliorating factors such as coinheritance either of α thalassaemia trait or of a mutation or polymorphism that leads to enhanced γ chain synthesis in conditions of haemopoietic stress (e.g. in *BCL11A*, *Xmn1-HBG2* or *HBS1L-MYB*). A polymorphism in the transcription factor gene, *CEBPE*, can also cause disease amelioration [338]. Those who have only a single abnormal β gene either have ‘dominant β thalassaemia’ or have coinherited mutations that aggravate the chain imbalance, such as homozygosity or heterozygosity for triple α or quadruple α . A rare cause of β thalassaemia intermedia is the occurrence of a somatic mutation during development with loss of one β gene from a proportion of haemopoietic cells in an individual who is heterozygous for a β thalassaemia mutation [332]. In a unique individual there was transition, during adult life, of typical heterozygous β thalassaemia to thalassaemia intermedia as a result of progressive somatic clonal segmental deletion that included the normal β globin gene cluster [339]. In some patients the explanation for a thalassaemia intermedia rather than a thalassaemia major or thalassaemia minor phenotype is not clear. Some genotypes are consistently associated with thalassaemia intermedia

whereas there are others that are sometimes associated with thalassaemia major and sometimes with thalassaemia intermedia.

In some communities, β thalassaemia intermedia is relatively common. For example, in Sardinia 10% of patient who are homozygotes or compound heterozygotes for β^0 thalassaemia have a thalassaemia intermedia phenotype, as a result of coinheritance of homozygous α^+ thalassaemia, non-deletional α thalassaemia or heterocellular HPFH [340]. In countries where haemoglobin E is common, compound heterozygosity for haemoglobin E and β thalassaemia makes thalassaemia intermedia a common phenotype.

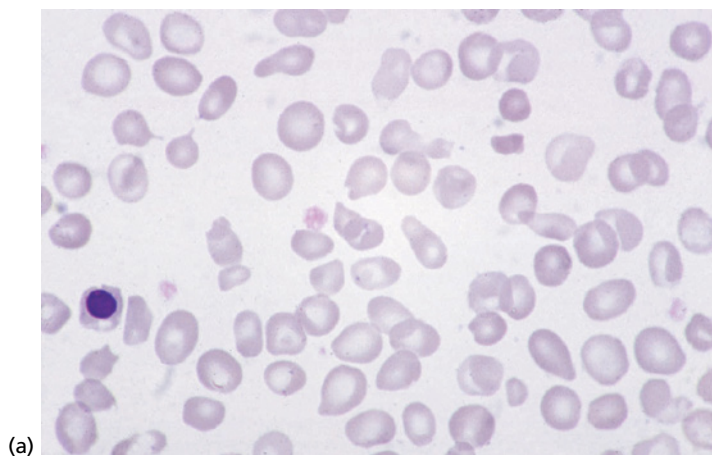
It has been suggested that in Mediterranean countries with a high incidence of β thalassaemia, prenatal diagnosis should include seeking to identify $\alpha\alpha$ and silent β thalassaemia due to $-101\text{ C} \rightarrow \text{T}$ in the partners of women with β thalassaemia trait, in order to predict the occurrence of β thalassaemia intermedia and offer informed choice regarding pregnancy outcomes [341]. Heterozygosity for both $\alpha\alpha$ and β thalassaemia leads to the clinical picture of thalassaemia intermedia in three-quarters of cases while compound heterozygosity for both $\alpha\alpha$ and $-101\text{ C} \rightarrow \text{T}$ silent β thalassaemia leads to a mild thalassaemia intermedia phenotype in 71% of instances [341]. Testing for $\alpha\alpha$ would be most likely to give relevant information since this was detected in 3.8% of partners of women with β thalassaemia trait whereas $-101\text{ C} \rightarrow \text{T}$

silent β thalassaemia was detected in only 0.2% [341].

Hydroxyurea/hydroxycarbamide is widely used in some countries to treat patients with β thalassaemia intermedia, including that due to β thalassaemia/haemoglobin E disease, to increase γ globin synthesis and reduce globin chain imbalance. Thalassaemia intermedia may respond to therapy with mitapivat, a stimulator of pyruvate kinase activity, with a rise in Hb and reduced bilirubin and LDH; this is currently being investigated in early-stage clinical trials [146]. Luspatercept, which enhances erythroid maturation, similarly leads to a rise of Hb in approaching 80% of the above two groups of patients [342]. The choice of treatments depends on the clinical picture, although if the Hb is consistently less than 100 g/l this may be taken as an indicator of likely benefit from either transfusion or the use of drugs to improve ineffective haemopoiesis [343].

Laboratory features

The Hb is very likely to decrease with age; in one study of 584 patients there was a mean of about 90 g/l in infancy in contrast to a mean of about 60 g/l by 50 years of age ($p < 0.001$) [344]. The blood film shows features similar to those of typical β thalassaemia trait but the abnormalities are more severe (Fig. 3.48). In addition to hypochromia, microcytosis, anisocytosis,



(a)

Fig. 3.48 Blood films from four patients with thalassaemia intermedia: (a) adult female with Hb 79 g/l, heterozygosity for β thalassaemia and triple alpha ($\beta\beta$ IVS1 nt five mutation, $\alpha\alpha\alpha/\alpha\alpha$) (with thanks to Dr N. Jackson); (Continued on p. 160.)

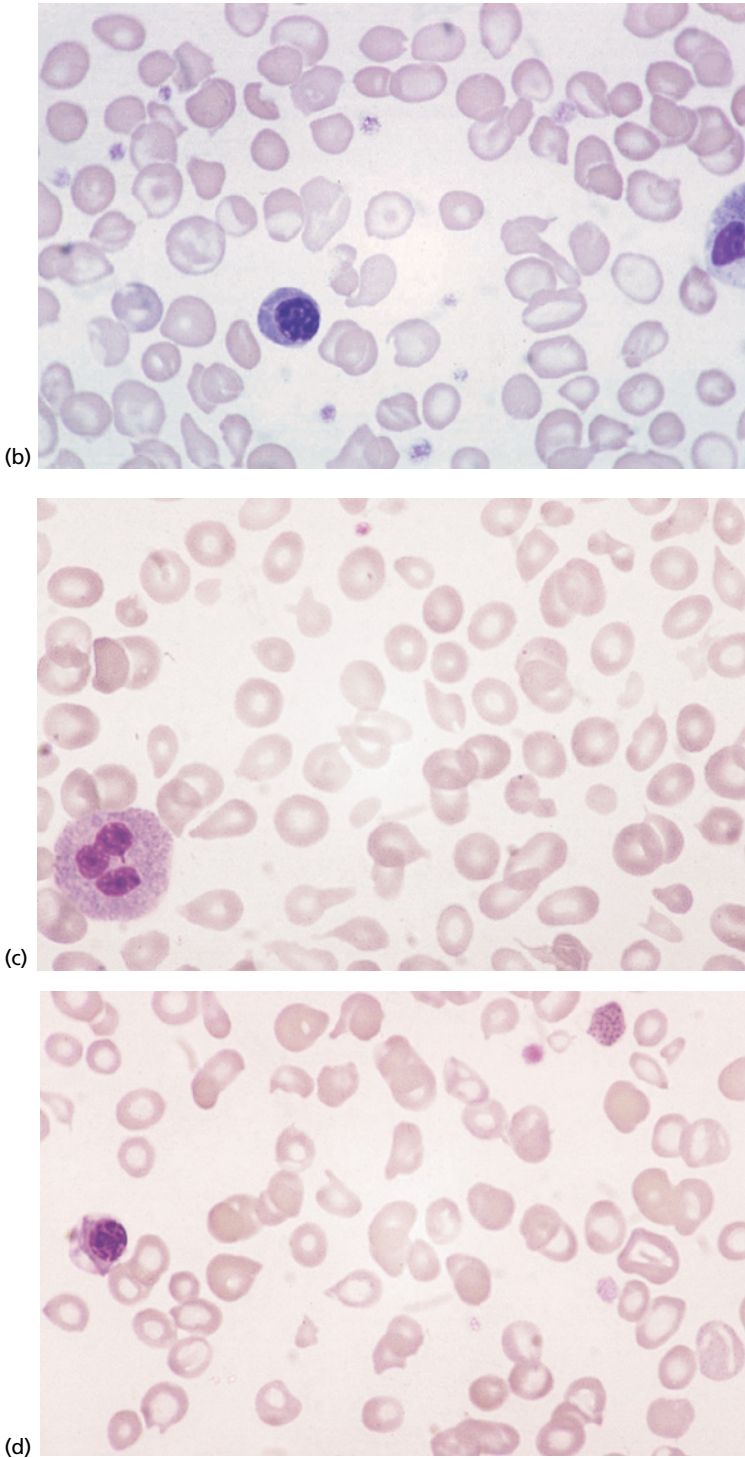
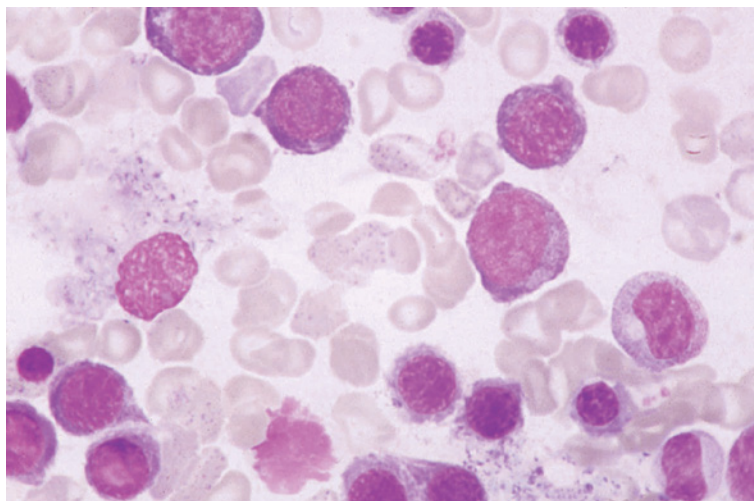


Fig. 3.48 *Continued.* (b) six-year-old girl with 8 cm splenomegaly and Hb 86 g/l with compound heterozygosity for β^0 thalassaemia and type 2 deletional hereditary persistence of fetal haemoglobin; (c) adult female with compound heterozygosity for β^0 and silent β thalassaemia trait, Hb 89 g/l, haemoglobin F 23%, haemoglobin A₂ 6.3% (genotype β 39 C→T, β -101 C→T); (d) adult female with homozygosity for very mild β^+ thalassaemia, the -29 A→G mutation. MGG $\times 100$.

Fig. 3.49 Bone marrow film from a patient with thalassaemia intermedia (same patient as Fig. 3.41a) showing erythroid hyperplasia and scanty ragged cytoplasm.



poikilocytosis and basophilic stippling there may be polychromasia and circulating erythroblasts. Circulating erythroblasts may contain α chain inclusions [345] and, following splenectomy, these can also be seen in erythrocytes. The findings on haemoglobin electrophoresis or HPLC are dependent on the precise underlying genetic defect (see Table 3.11). The haemoglobin A₂ percentage is likely to be elevated somewhat more than in β thalassaemia trait and the haemoglobin F is elevated. Serum erythropoietin is increased, particularly when haemoglobin F (a high affinity haemoglobin) is more than 50%. The bone marrow aspirate shows abnormalities of erythropoiesis that are more severe than those of β thalassaemia trait (Fig. 3.49).

The haematological features may be altered by therapy. Response to hydroxycarbamide can be associated with a rise in Hb, MCV and haemoglobin F percentage and a fall in the number of circulating erythroblasts [346].

Iron stores are increased, with serum iron, transferrin saturation and ferritin are elevated. In comparison with β thalassaemia major, serum ferritin underestimates the degree of iron overload. If appropriate magnetic resonance imaging is not available to quantitate hepatic and cardiac iron, a serum ferritin of 800 ng/ml can be used as an indication for iron chelation therapy with therapy being interrupted if the level falls to 300 ng/ml and the dose of chelating agent escalated if the level rises to 2000 ng/ml

[347]. However, it should be noted that hepatic steatosis is not uncommon in thalassaemia intermedia and leads to higher serum ferritin in comparison with patients without steatosis [348].

Problems in the diagnosis of β thalassaemia intermedia

Misdiagnosis has occurred when anaemia is due to coinheritance of a mutation leading to a red cell membrane abnormality, for example in *ANK1*, *SLC4A1* or *SPTA1*, with β thalassaemia heterozygosity [349].

β thalassaemia major

β thalassaemia major refers to patients who have inherited two significant β thalassaemia alleles causing significant globin chain imbalance, who are dependent on blood transfusions to maintain life beyond early childhood. The alternative designation, transfusion-dependent thalassaemia, is often used, reflecting how patients are treated rather than any particular genotype or pathophysiology. It has been estimated that in the UK there are 20–30 births per year of babies with β thalassaemia major. Very rarely, heterozygotes for β thalassaemia have the clinical phenotype of β thalassaemia major as a result of coinheritance of extra copies of the α gene; one such patient was homozygous for

$\alpha\alpha$ and another was homozygous for $\alpha\alpha\alpha$ [350]. Even more rarely there is evolution of thalassaemia minor to thalassaemia major as a result of uniparental disomy of 11p15 sequences [351]. This can be the result of mosaic uniparental disomy with progressive clonal selection [352]. Equally rarely β thalassaemia major develops in adult life as a result of a somatic deletion in an individual with β thalassaemia heterozygosity [339]. The phenotype of β thalassaemia major can also result from compound heterozygosity for a β thalassaemia allele and a 'thalassaemic haemoglobinopathy', such as haemoglobin E or the less common haemoglobin Malay [150], and haemoglobin E/ β thalassaemia is probably the most common genotype causing severe thalassaemia in the world.

Patients with β thalassaemia major have both ineffective erythropoiesis and a considerably shortened red cell life span (20 days or less) leading to severe anaemia. Ineffective haemopoiesis results from damage to erythroblasts, both by free α chains and from α chain precipitates. Free α chains are normally bound by the erythroid-specific molecular chaperone, α haemoglobin stabilising protein, but when its binding capacity is exceeded they undergo auto-oxidation to highly damaging α -hemichromes (α globin monomers containing oxidised ferric iron) and generate reactive oxygen species [353]. The α -hemichromes cause clumping of band 3 protein leading to anti-band 3 antibody-mediated clearance of erythroblasts and erythrocytes. Reactive oxygen species oxidise proteins and membrane lipids, thus contributing to haemolysis and also cause ineffective erythropoiesis by activation of apoptosis and by degradation of GATA1 [353]. Haemolysis is both intravascular and extravascular, the former leading to nitric oxide scavenging and platelet activation. Reduced serum haptoglobin and haemopexin reflect the intravascular element of the haemolysis. The disease usually presents in the first year of life, from the age of three months onwards. Initial presentation is usually with failure to thrive, episodes of infection or abdominal enlargement. In the absence of treatment, thalassaemia follows a severe and



Fig. 3.50 The face of a child with β thalassaemia major showing frontal bossing, prominence of the maxilla and displacement of the teeth. (By courtesy of the late Professor Harry Smith.)

progressive course, usually resulting in death in the first few years of life. There is markedly increased erythropoiesis, both in an expanded bone marrow compartment and at extramedullary sites. The expansion of haemopoietic bone marrow leads to bony deformity, particularly in the skull and facial bones with frontal bossing, deformity of the facial bones, displacement of the teeth and a 'hair-on-end' appearance on skull radiography (Figs 3.50 and 3.51). There is sometimes bone pain, for example in the jaw and the vertebral column, and tenderness and an increased incidence of fractures consequent on thinning of cortical bone. Erythropoiesis at extramedullary sites leads to gross hepatomegaly and splenomegaly (Fig. 3.52). The splenomegaly is associated with increased trapping of abnormal red cells in the spleen. The splenomegaly can also lead to hypersplenism with an expanded plasma volume and pooling of red cells and platelets in the spleen. Rarely extramedullary haemopoiesis leads to spinal cord compression [354]. Ineffective haemopoiesis and shortened red cell life span lead to mild jaundice and an increased incidence of gallstones. There is wasting of the limbs and

Fig. 3.51 Skull radiograph of a child with β thalassaemia major showing a 'hair-on-end' appearance as a consequence of marked erythroid hyperplasia. (Reproduced with permission from Hoffbrand and Steensma (2020)/John Wiley & Sons.)

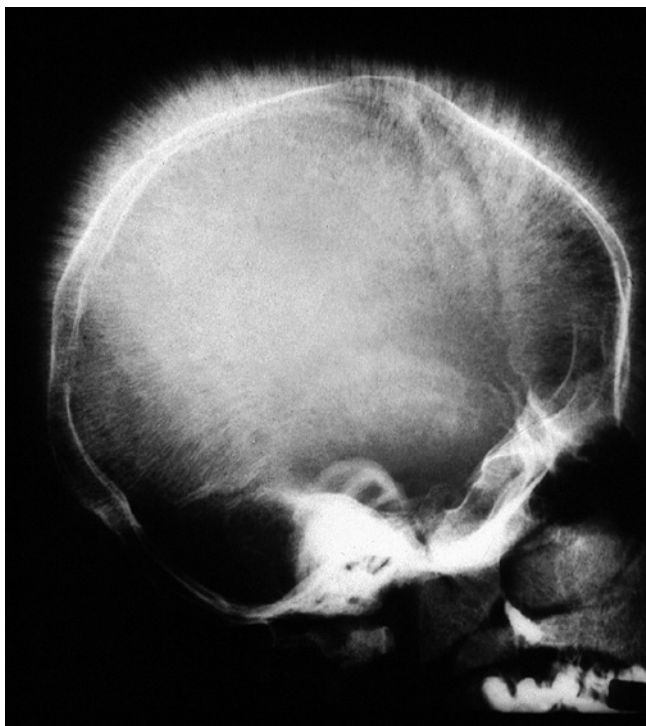


Fig. 3.52 An undertransfused child with β thalassaemia major showing abdominal distension and an everted umbilicus caused by gross hepatosplenomegaly; there is also wasting of limbs.



stunting of growth. Leg ulcers can occur. Severe anaemia can cause high output cardiac failure. Anaemia also causes growth retardation. There can be a hypercoagulable state as a result of damage to the red cell membrane and nitric oxide scavenging; prior splenectomy can contribute. There is a resultant increased incidence of venous thromboembolism, portal vein thrombosis and pulmonary hypertension. Pulmonary hypertension is prognostically adverse [355]. Pulmonary fibrosis, attributable to iron deposition, can also contribute to pulmonary hypertension and hypoxia. Regularly transfused and iron-chelated patients can have hypercalciuria with an increased incidence of kidney stones [356]. Cognitive impairment has been reported [323]. There is an increased incidence of headaches (in a study group of β thalassaemia major and intermedia and haemoglobin E/ β thalassaemia), which correlates with white matter lesions on magnetic resonance imaging [357]. There is a significant prevalence of hepatic steatosis, 20% in one study, which correlates with body mass index and may progress to fibrosis [358]. Avascular necrosis of the hips has also been reported [359].

Many of these adverse effects of β thalassaemia major can be largely avoided by an appropriate blood transfusion and iron chelation programme. Regular transfusions lead to serious iron overload unless chelation therapy is given. Iron overload in turn can lead to cardiac and hepatic damage, hypopituitarism (contributing to growth retardation), hypogonadotropic hypogonadism with delayed puberty, diabetes mellitus, hypothyroidism and hypoparathyroidism (often subclinical). Adrenal insufficiency of central origin may be subclinical and may only become apparent under stress [360]. Cardiac damage can manifest as left ventricular dilation with a reduced ejection fraction or as diastolic dysfunction with restrictive filling [361]. There can be valvular disease, atrial flutter or fibrillation, ventricular tachycardia and heart failure. Males are more likely to have cardiac complications than females with the same degree of iron overload [362]. Liver disease includes fibrosis, cirrhosis and hepatocellular carcinoma with possible interaction with

transfusion-transmitted hepatitis B or C in some countries. There is an increased incidence of papillary carcinoma of the thyroid. There may also be vitamin D deficiency and hypercalciuria of uncertain mechanism [363]. There is an increased prevalence of renal calculi, correlating with hypercalciuria and reduced bone density [364]. There is an association with myelolipoma, a rare benign tumour composed of mature adipose tissue and islands of haemopoietic cells [365].

In the absence of treatment, children with homozygosity for β^0 thalassaemia usually die at 3–4 years of age whereas those with β^+ homozygosity may survive to late childhood [291]. With iron chelation regimes becoming less arduous, it is more feasible to start transfusion earlier, thus reducing the frequency of alloimmunisation. With optimal treatment, life expectancy and quality of life should approach that of the non-thalassaemic population, although this is not possible for most patients in the world who do not have access to safe blood transfusions or effective iron chelation.

Laboratory features

In the absence of transfusion, the Hb, RBC, Hct, MCV, MCH and MCHC are reduced and RDW is increased. The Hb is usually in the range of 30–70 g/l, the MCV 50–60 fl and the MCH 12–18 pg. The blood film (Fig. 3.53) shows marked anisocytosis, poikilocytosis (including fragments and teardrop poikilocytes), hypochromia and microcytosis. Basophilic stippling, Pappenheimer bodies and target cells may be noted. Following splenectomy, erythrocytes may contain α chain inclusions [345]. Circulating nucleated red cells showing defective haemoglobinisation and dyserythropoietic features are present. The total white cell count and the neutrophil count are increased. In children with massive splenomegaly, hypersplenism leads to aggravation of the anaemia and leucopenia, neutropenia and thrombocytopenia. The percentage of reticulocytes is increased. The absolute reticulocyte count is stated to be rarely high although it tends to increase after splenectomy [224]. Serum levels of both haptoglobin

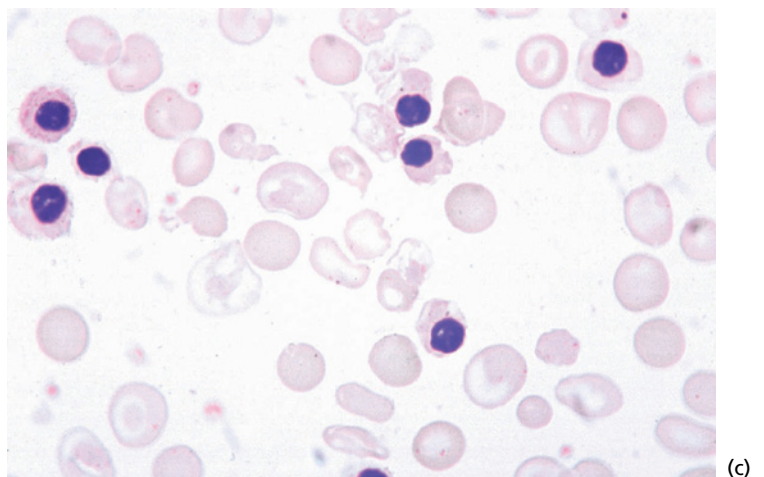
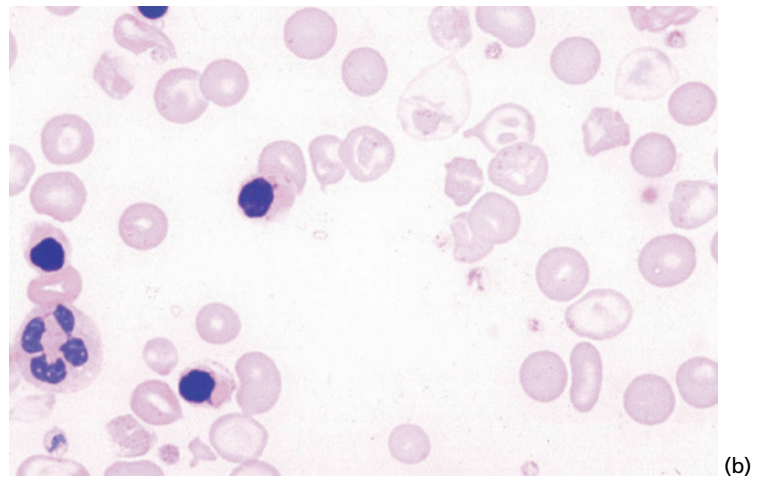
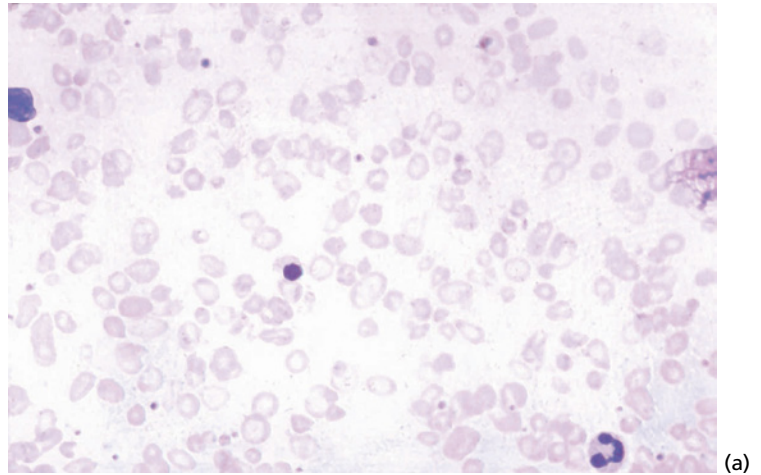


Fig. 3.53 Blood films of four patients with β thalassaemia major; patients (a) and (b) were being transfused; patient (c) had not been transfused for the previous three months because of the development of red cell alloantibodies; all these patients had been splenectomised; α chain precipitates are clearly seen in all patients; patient
(Continued on p. 166.)

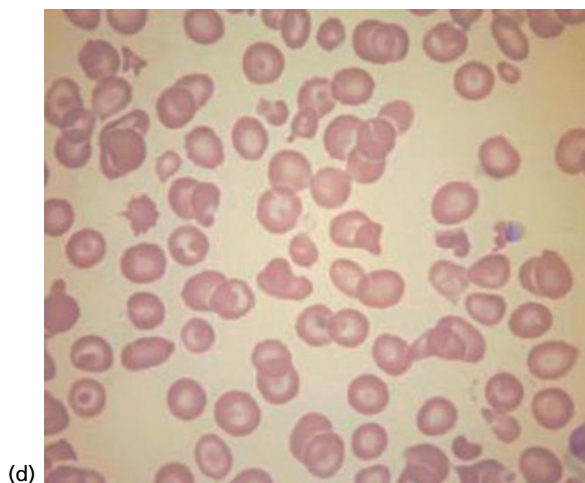


Fig. 3.53 *Continued.* (d) was a baby of four months of age who had never been transfused.

and haemopexin are reduced, reflecting the intravascular element of the haemolysis.

The concentrations of protein C, protein S and antithrombin are often reduced [366].

If the spleen has been removed, the usual features of hyposplenism are present: Howell–Jolly bodies, target cells, lymphocytosis, thrombocytosis and giant platelets. Pappenheimer bodies are very prominent and nucleated red cells are markedly increased. After splenectomy the red cells may show inclusions with the same staining characteristics as haemoglobin; these stain supravitaly with methyl violet. They represent α chain precipitates. Such inclusions are present in much smaller numbers in patients who have not been splenectomised. α chain precipitates may also be detectable in circulating nucleated red cells. Following splenectomy, the blood film may show leptocytes, which are very flat cells with little reduction in cell diameter but striking hypochromia.

The bone marrow aspirate (Fig. 3.54) shows gross erythroid hyperplasia. There is quite severe dyserythropoiesis with nuclear lobulation and fragmentation, basophilic stippling, defective haemoglobinisation and the presence of α chain precipitates. Actively phagocytic macrophages are prominent and pseudo-Gaucher cells are present. Iron stores are increased. Ultrastructural examination shows that α chain precipitates are often present in profiles of late polychromatic erythroblasts and

in a low percentage of profiles of early polychromatic erythroblasts; arrest in G1, seen quite frequently in the latter population, may be attributable to the presence of free α chains [367]. Erythroblasts, particularly those containing α chain precipitates, show other structural abnormalities in both cytoplasm and nucleus.

In the case of homozygotes or compound heterozygotes for β^0 thalassaemia ($\beta^0\beta^0$), techniques such as haemoglobin electrophoresis, IEF and HPLC show only haemoglobin F and haemoglobin A₂ (Fig. 3.55). When there is homozygosity for β^+ thalassaemia ($\beta^+\beta^+$) or compound heterozygosity for β^0 and β^+ thalassaemia ($\beta^0\beta^+$) haemoglobin A is also present, in variable amounts, depending on the severity of the defect of various β^+ thalassaemia alleles. In β thalassaemia major, the haemoglobin A₂ percentage may be normal, elevated or, occasionally, reduced. A Kleihauer test shows that haemoglobin F is greatly increased.

Biochemical tests show increased bilirubin, increased urinary urobilinogen and hyperuricaemia. Serum soluble transferrin receptor (an indicator of erythropoietic activity) is increased greatly and erythropoietin levels are high. Serum haptoglobin is greatly reduced or absent, as a result of intravascular haemolysis, the level correlating inversely with serum transferrin receptor [368]. Serum haemopexin is similarly decreased. Free haemoglobin may be detectable in the plasma and methaemalbumin may be present.

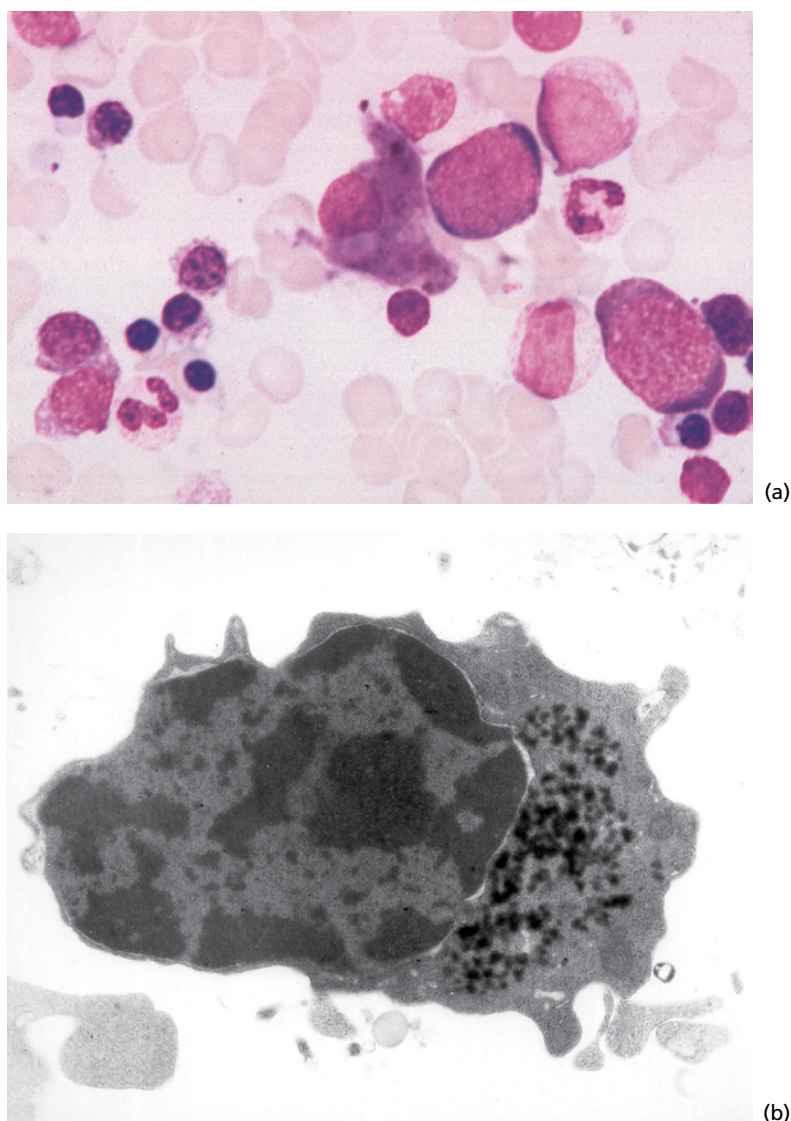


Fig. 3.54 Bone marrow aspirates from two patients with β thalassaemia major: (a) an MGG-stained film showing erythroid hyperplasia and a debris-laden macrophage; (b) ultrastructural examination showing α chain deposits (by courtesy of the late Professor Sunitha N. Wickramasinghe).

$\delta\beta$, $\gamma\delta\beta$ and $\epsilon\gamma\delta\beta$ thalassaemias

$\delta\beta$ and $\Delta\gamma\delta\beta$ thalassaemias

$\delta\beta^0$ or $(\delta\beta)^0$ (delta beta zero) thalassaemia (sometimes also designated $\text{C}_{\gamma}\Delta\gamma(\delta\beta)^0$ thalassaemia) results from deletion of both δ and β genes but with preservation of the γ genes. $\Delta\gamma\delta\beta^0$ or $(\Delta\gamma\delta\beta)^0$ thalassaemia (sometimes also designated $\text{C}_{\gamma}(\Delta\gamma\delta\beta)^0$ thalassaemia) results from deletions of the $\Delta\gamma$, δ and β genes. The phenotype of heterozygotes of both resembles that of

β thalassaemia trait but the haemoglobin A_2 percentage is not increased; since one δ gene has been lost it might be expected that haemoglobin A_2 would be reduced but in fact it is often normal [369]. The blood film features (Fig. 3.56) are very similar to those of β thalassaemia trait. Haemoglobin F is consistently elevated, usually between 5% and 20% (Figs 3.57 and 3.58) [370]. The distribution of haemoglobin F, best observed by flow cytometry, is heterocellular. Because of the increased synthesis of haemoglobin F, homozygotes and

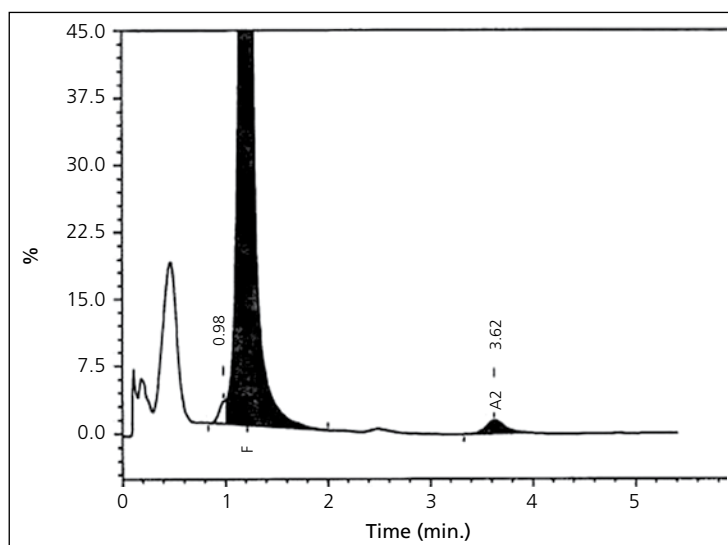


Fig. 3.55 HPLC chromatogram (Bio-Rad Variant II) in a baby of four months of age with β thalassaemia major (same case as Fig. 3.46d); the haemoglobin A_2 was increased at 1.4% but otherwise the chromatogram is not distinguishable from that of a newborn premature neonate; from left to right, the peaks are post-translationally modified haemoglobin F, haemoglobin F (black) and haemoglobin A_2 .

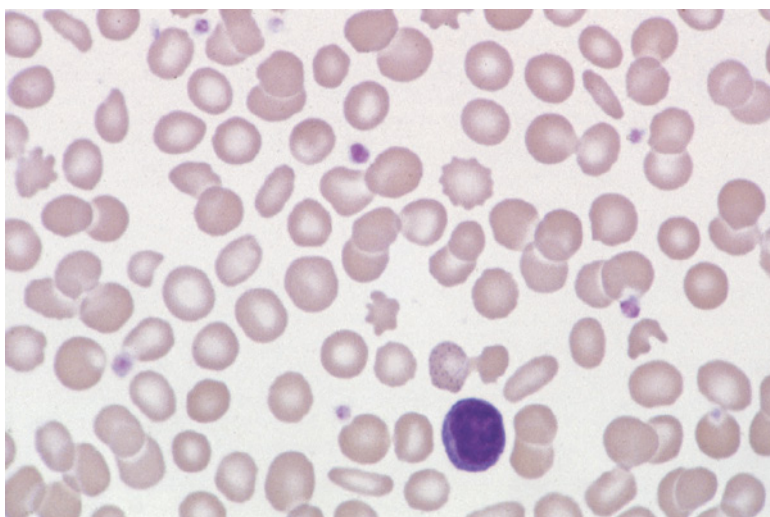


Fig. 3.56 Blood film of an adult male with $\delta\beta$ thalassaemia trait; the red cell indices were RBC $6.04 \times 10^{12}/l$, Hb 140 g/l, Hct 0.42, MCV 69 fl, MCH 23.2 pg, MCHC 334 g/l. MGG $\times 100$.

compound heterozygotes with a severe β^+ or β^0 mutation may have thalassaemia intermedia rather than thalassaemia major. Homozygotes for $\delta\beta$ or $\alpha\gamma\delta\beta$ thalassaemia generally have the clinical and haematological features of thalassaemia intermedia (Fig. 3.59); they have 100% haemoglobin F (Fig. 3.60) and necessarily a pancellular distribution [371]. In $\delta\beta$ thalassaemia homozygotes, $\gamma\gamma$ and $\alpha\gamma$ globin chains are present in similar amounts whereas homozygotes for $\alpha\gamma\delta\beta$ thalassaemia have only $\gamma\gamma$ globin chains. Heterozygotes for either of these types

of thalassaemia usually have splenomegaly and an Hb of 80–130 g/l. The MCV may be reduced or low normal and the MCH reduced or normal.

There are at least 10 mutations giving rise to $\delta\beta^0$ thalassaemia. This type of thalassaemia is observed in many ethnic groups including some Mediterranean (Italians, Greeks and Turks), black, Spanish and Japanese populations [188]. There are at least 12 mutations giving rise to $\alpha\gamma\delta\beta$ thalassaemia. This type of thalassaemia also occurs in many ethnic groups including Indian and Chinese populations.

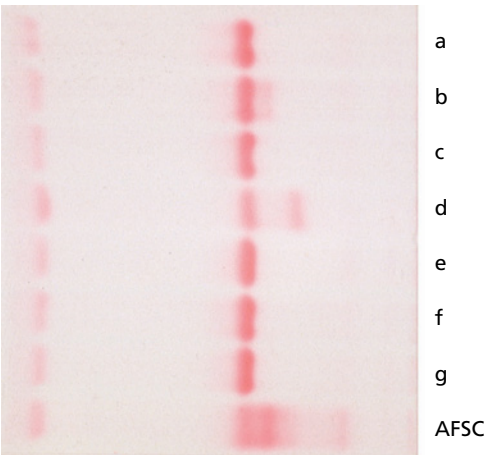


Fig. 3.57 Haemoglobin electrophoresis on cellulose acetate at alkaline pH (lane b) of an adult male with $\delta\beta$ thalassaemia (same patient as Fig. 3.56) showing increased haemoglobin F; AFSC indicates a control sample containing haemoglobins A, F, S and C.

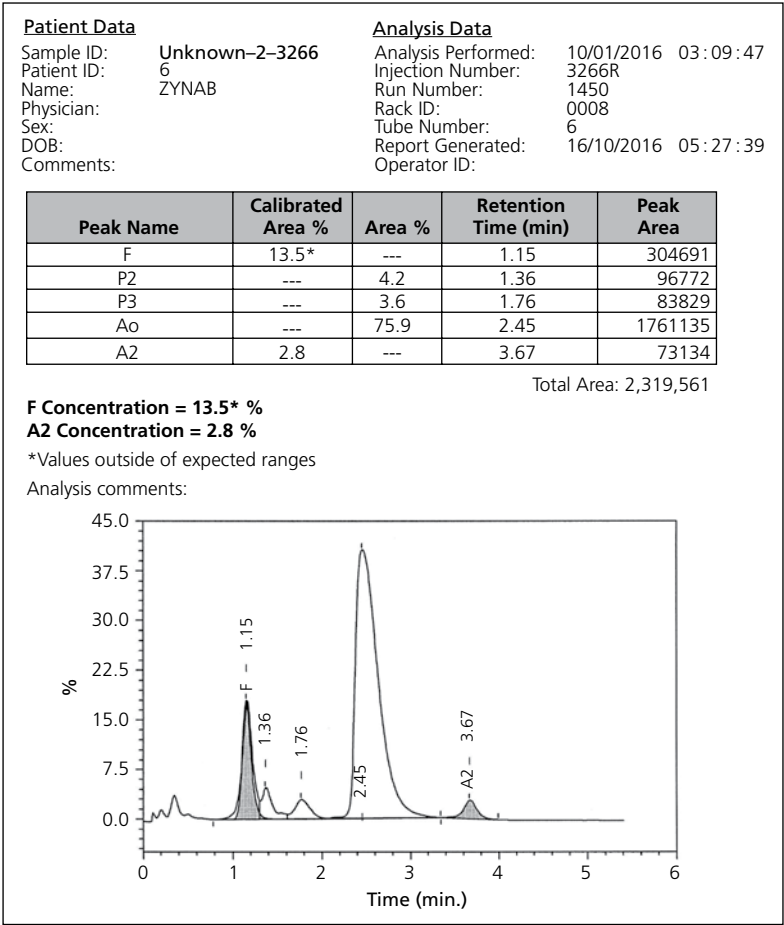


Fig. 3.58 HPLC (Bio-Rad Variant II) of a 10-year-old Iraqi girl with heterozygosity for $\delta\beta$ thalassaemia. The red cell indices were RBC $5.41 \times 10^{12}/l$, Hb 105 g/l, Hct 0.38, MCV 64 fl, MCH 19.4 pg, MCHC 303 g/l, red cell distribution width (RDW) 32%. Three other heterozygotes in the family had haemoglobin A₂ of 2.7, 2.9 and 2.9% with haemoglobin F of 9.8, 6 and 12.6%. (With thanks to Dr Hadeel Ibrahim.)

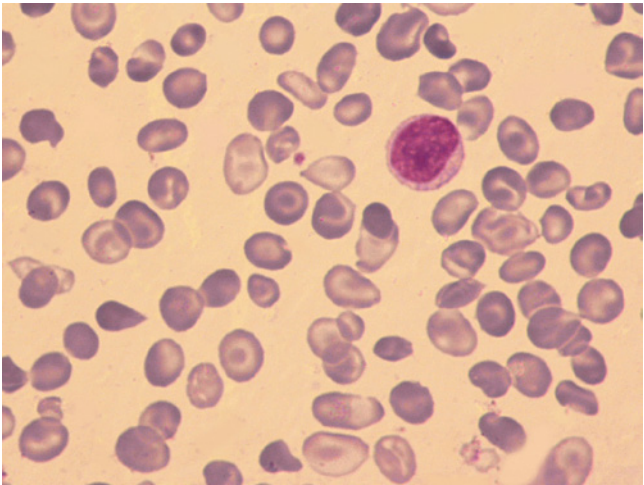


Fig. 3.59 Blood film of an 11-year-old Iraqi girl with homozygosity for $\delta\beta$ thalassaemia. The red cell indices were RBC $5.30 \times 10^{12}/l$, Hb 105 g/l, Hct 0.313, MCV 59 fl, MCH 19.8 pg, MCHC 335 g/l, RDW 32.9%. MGG $\times 100$. (With thanks to Dr Hadeel Ibrahim.)

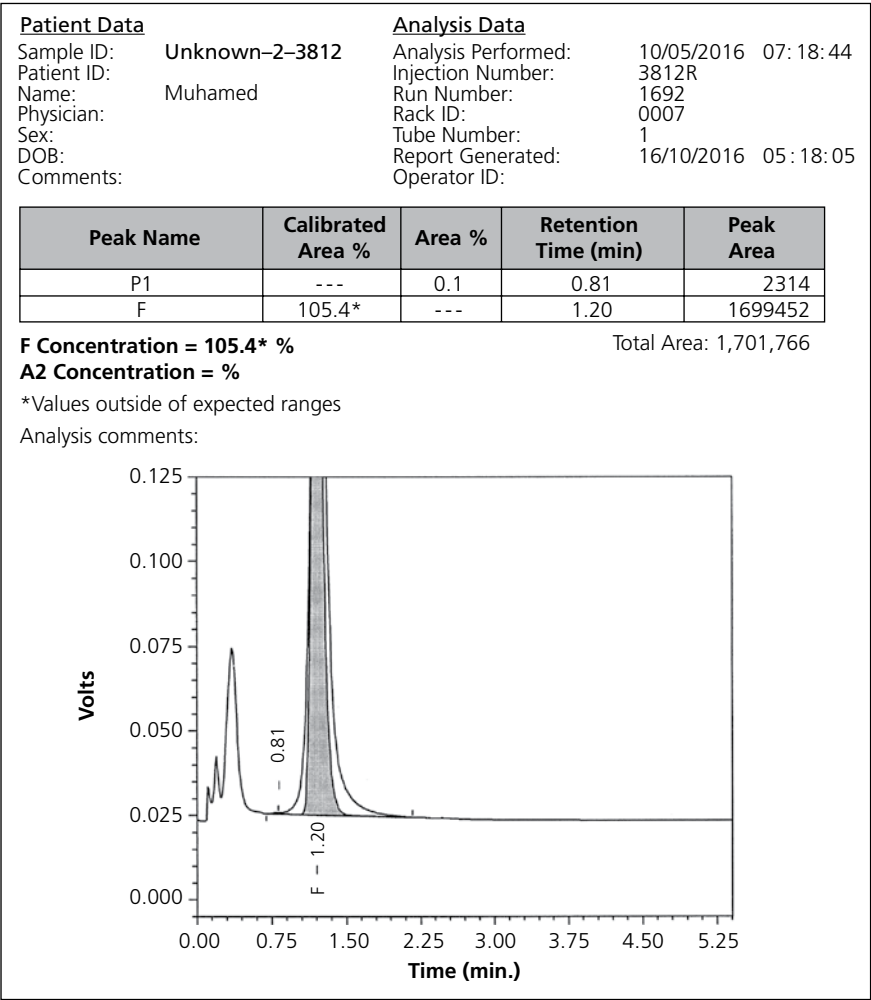


Fig. 3.60 HPLC (Bio-Rad Variant II) of an 11-year-old Iraqi girl with homozygosity for $\delta\beta$ thalassaemia (same patient as Fig. 3.52) showing an increase of acetylated haemoglobin F and F_{α_2} . (With thanks to Dr Hadeel Ibrahim.)

An unusual molecular mechanism underlying $\delta\beta$ thalassaemia is a $\delta\beta$ fusion gene, observed in a Senegalese family, that results in a $\delta^0\beta^+$ thalassaemia with the δ promoter controlling β chain synthesis [372]. The heterozygote described had thalassaemia trait with a normal haemoglobin A_2 percentage and 2.7% haemoglobin F. It is more usual for $\delta\beta$ fusion genes to lead to synthesis of haemoglobin Lepore (see earlier).

The Sardinian type of ' $\delta\beta$ thalassaemia trait' is actually a phenocopy of $\delta\beta$ thalassaemia trait caused by coinheritance (in *cis*) of the codon 39 nonsense mutation that is a common cause of β^0 thalassaemia in the Mediterranean area plus a mutation of the $^A\gamma$ promoter leading to overproduction of γ chain. There are thalassaemic indices with normal haemoglobin A_2 and 15–20% haemoglobin F [9], which is very largely $\alpha_2^A\gamma_2$. In one described homozygote, there was microcytosis but the Hb was normal and the condition was clinically silent; there was 99.8% haemoglobin F and 0.2% haemoglobin A_2 [373]. Another phenocopy, initially described in Corfu, is caused by coinheritance of a deletion, extending downstream from the $\psi\beta$ gene and encompassing the δ gene, and a point mutation in the β gene. Heterozygotes have the phenotype of $\delta\beta$ thalassaemia trait while homozygotes have almost 100% F, traces of haemoglobin A and no haemoglobin A_2 [9].

Haemoglobin Lepore (see earlier) can be regarded as a type of $\delta\beta^+$ thalassaemia since there is a reduced rate of synthesis of both δ and β chains.

The red cell indices are important in distinguishing between $\delta\beta$ thalassaemia trait and HPFH. However, if a patient with HPFH also has α thalassaemia (with deletion of two α genes) the indices will be significantly abnormal. DNA analysis is needed to make a reliable distinction.

$\epsilon\gamma\delta\beta$ thalassaemias

There are at least 20 mutations that either delete the entire β gene cluster (at least 14 examples) or inactivate all genes of the cluster because the upstream regulatory *LCRB* is deleted (at least six examples) [374, 375]. When *LCRB* is deleted,

all genes of the β cluster may be intact (Hispanic deletion) or there may also be deletion of ϵ and $^C\gamma$ leaving $^A\gamma$, δ and β intact but inactivated [376] or deletion of ϵ only leaving $^C\gamma$, $^A\gamma$, δ and β intact but inactivated (English II) [374]. In the case of one large deletion, there was also dysmorphism and mild intellectual disability, possibly related [377]. This type of thalassaemia is correctly referred to as $\epsilon\gamma\delta\beta$ or as $\epsilon^C\gamma^A\delta\beta$ thalassaemias but more commonly the term $\gamma\delta\beta$ (gamma delta beta) thalassaemia is used. Some cases result from *de novo* mutation. All are rare and are recognised only in heterozygotes. The homozygous state would be incompatible with fetal life. In the neonatal period, heterozygotes are characterised by haemolysis, possibly with erythroblastosis, hepatomegaly and splenomegaly; there may be a need for blood transfusion at birth and during the first six months of life. Occasional cases have had life-threatening neonatal anaemia or have required intrauterine transfusion [376, 378]. The blood film shows microcytosis and sometimes basophilic stippling (Fig. 3.61) and the reticulocyte count may be increased. Thereafter the phenotype resembles that of β thalassaemia trait, although there may be anaemia and microcytosis and hypochromia tend to be more severe [374, 375]. Anaemia may become severe during pregnancy [379]. There can be mild haemolysis [375]. There is no elevation of haemoglobin F or A_2 . Historically, the diagnosis was made by globin chain synthesis studies, which have now been largely replaced by measurements of the different globin mRNA levels. In practice, the diagnosis is now made by DNA analysis, such as MLPA, looking for large deletions, and by next-generation sequencing approaches able to detect copy number variants. Deletion of the β gene can also be detected by fluorescence *in situ* hybridisation [378].

Coinheritance of $\epsilon\gamma\delta\beta$ and triple α can lead to hydrops fetalis [379].

δ thalassaemia

Delta or δ thalassaemia is of no clinical significance since it only affects synthesis of haemoglobin A_2 . It is, however, of significance in

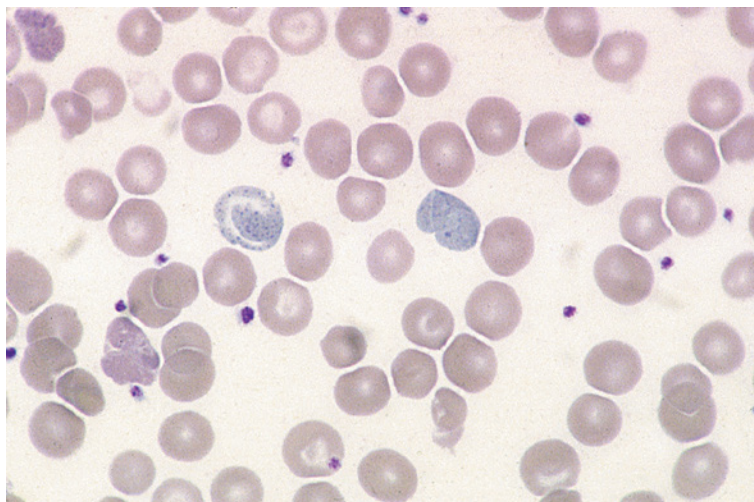


Fig. 3.61 Blood film in a neonate with $\gamma\delta\beta$ thalassaemia trait showing microcytosis and basophilic stippling. MGG $\times 100$. (With thanks to Dr Manju Bhavnani.)

relation to diagnosis of β thalassaemia trait since inheritance of δ thalassaemia in *cis* or in *trans* to β thalassaemia means that the haemoglobin A_2 will not be elevated and the diagnosis of β thalassaemia heterozygosity may be missed. Both δ^0 and δ^+ mutations exist. There are also δ chain variants that are unstable or, like haemoglobin A_2 -Yialousa, synthesised at a reduced rate without there necessarily being an abnormal peak or band [380]. Heterozygotes and homozygotes for δ^+ thalassaemia have a reduced percentage of haemoglobin A_2 while haemoglobin A_2 is reduced in δ^0 heterozygotes and absent in homozygotes (Figs 3.62 and 3.63). For other inherited and acquired causes of reduced haemoglobin A_2 , which should be considered in the differential diagnosis, see Tables 3.12 [369, 381, 382] and 6.3.

Mutations responsible for δ thalassaemia include a large deletion, point mutations and frameshift mutations. The non-deletional mutations can produce a premature STOP codon or interfere with either transcription or RNA processing or translation. There are also structural haemoglobin A_2 variants synthesised at a reduced rate, thus leading to a 'thalassaemic haemoglobinopathy'. The so-called Corfu $\delta\beta$ thalassaemia (see earlier) is actually a phenotype of $\delta\beta$ thalassaemia caused by coexistence of δ^0 and β^+ thalassaemia.

γ thalassaemia

Gamma or γ thalassaemia refers to a reduced rate of synthesis of γ chain and therefore of haemoglobin F. This condition is manifest maximally during intrauterine life and, since there are normally four γ genes, clinical sequelae are likely to be minor. One recognised cause is deletion of the 3' part of the γ gene and the 5' part of the γ gene with production of a $\gamma\gamma$ fusion gene under the control of a γ promoter [383]. Rarely, this may be an explanation for severe fetal and neonatal anaemia that fully resolves in the first few months of life, and diagnosis is made by DNA analysis, usually direct sequencing of the γ globin genes.

Hereditary persistence of fetal haemoglobin (HPFH) and other inherited causes of an increased proportion of haemoglobin F

In adult life haemoglobin F is usually quite a low percentage of total haemoglobin and is confined to a small proportion of cells, designated F cells. However, in many ethnic groups 10–15% of individuals have a slight increase in the percentage of haemoglobin F and the percentage of F cells. This is the most common form of HPFH.

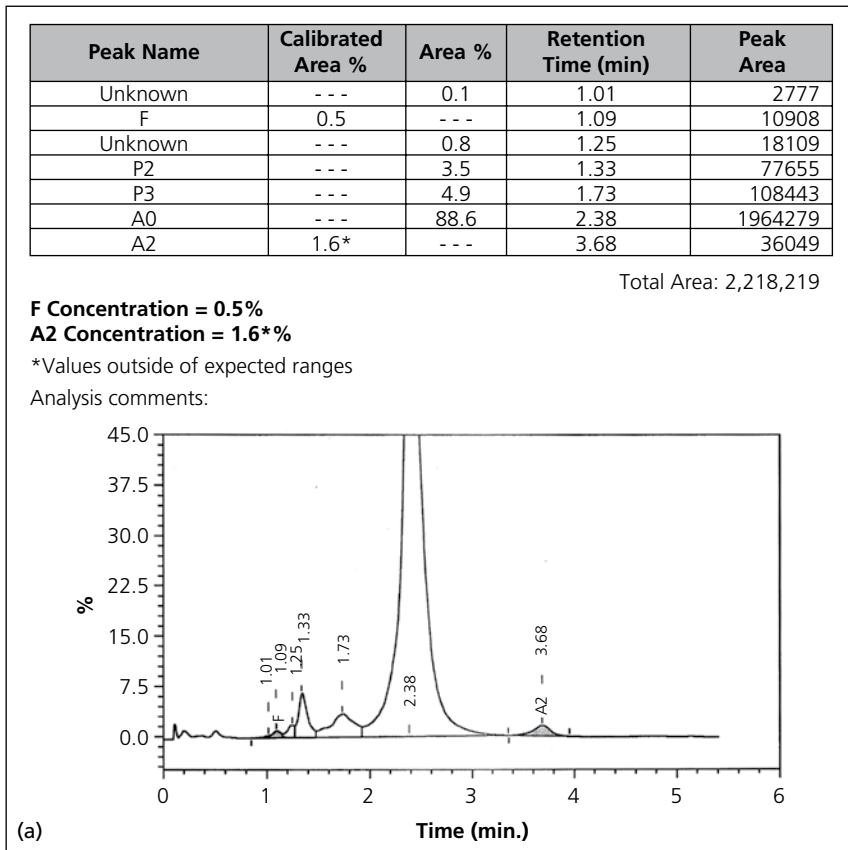


Fig. 3.62 Investigations of a patient with δ thalassaemia (likely to be either heterozygosity for δ^0 thalassaemia or homozygosity for δ^+ thalassaemia) diagnosed on the basis of there being no other explanation for the low haemoglobin A_2 ; (a) HPLC (Bio-Rad Variant II); (Continued on p. 174.)

HPFH can be defined as an inherited characteristic in which there is an increased proportion of haemoglobin F, persisting beyond infancy, with little or no imbalance of chain synthesis and normal red cell indices. Definitions of an increased haemoglobin F percentage in this context have included 1% or above [384] and 2% or above [385]. HPFH has also been defined in terms of the proportion of F cells with less than 4% F cells being classified as normal, 4–8% as equivocal and more than 8% as diagnostic [384]. There are other inherited (Table 3.13) [250, 386–394] and acquired (see Table 6.2) causes of an increased proportion of haemoglobin F. Conversely, a reduced percentage of haemoglobin

bin F is observed in infants with Down syndrome up to 60 days of life [381]. HPFH can result from deletions within the β globin cluster or from mutations or polymorphisms of regulatory genes, either on chromosome 11 or on other chromosomes. The deletional HPFHs result from relatively large deletions that include the δ and β genes. The critical difference between deletional HPFH and similar deletions that cause β^0 thalassaemia may be that in the former there is deletion of a globin gene silencer located 5' to the δ globin gene [395]. Since there is no β gene on the affected chromosome, deletional HPFH behaves as if it were an allele of the β globin gene; both homozygotes for HPFH and

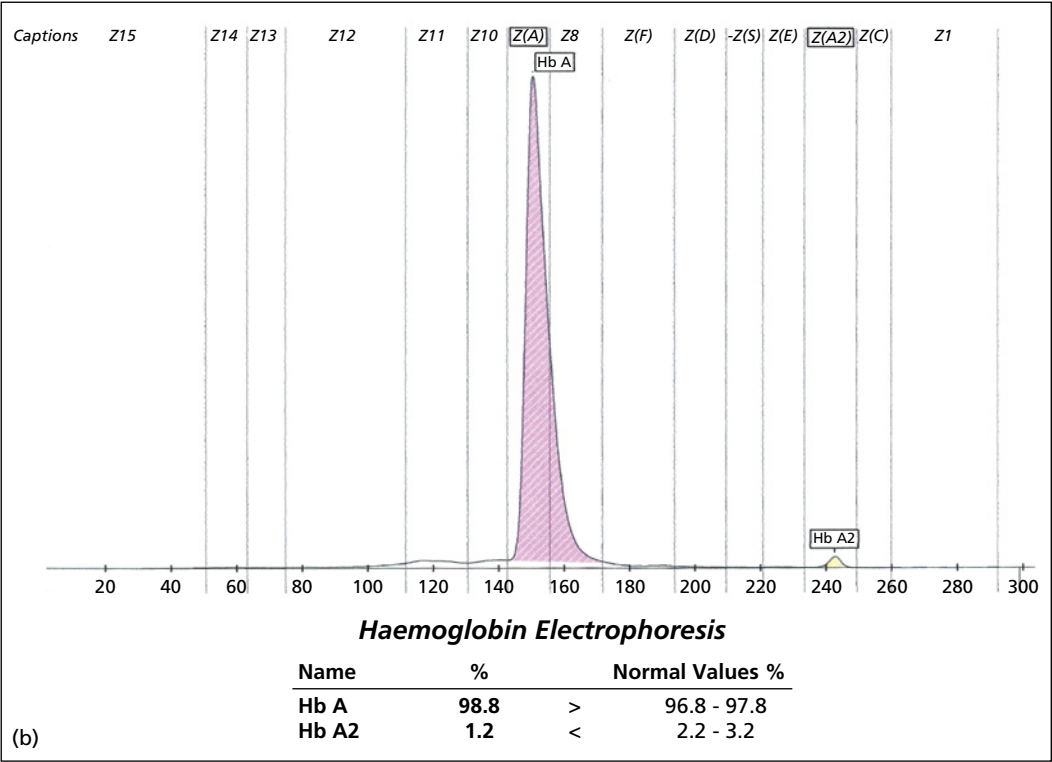


Fig. 3.62 Continued. (b) capillary electrophoresis (Sebia Capillarys).

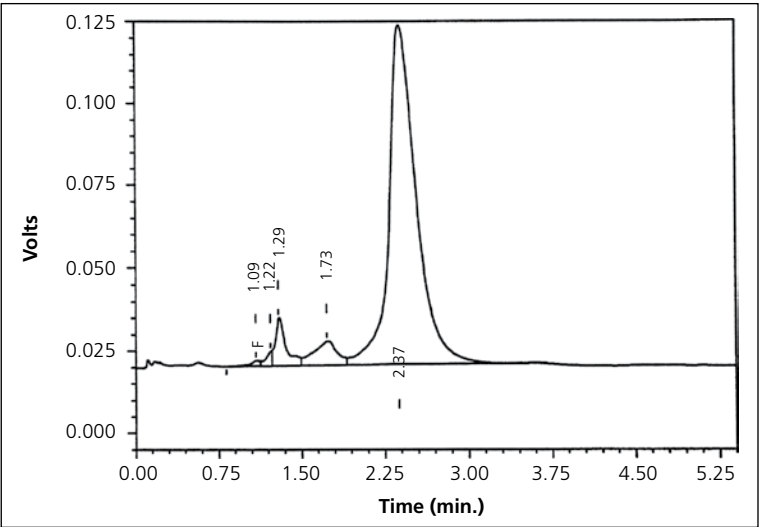


Fig. 3.63 HPLC chromatogram (Bio-Rad Variant II) in δ^0 thalassaemia homozygosity showing a total absence of haemoglobin A₂.

compound heterozygotes for HPFH and a β chain variant totally lack haemoglobin A. Synthesis of haemoglobin F is upregulated because γ genes are brought into proximity to an enhancer 3' to the deletion [334]. The non-deletional HPFHs are a heterogeneous group of

disorders some of which are allelic to the β gene and some of which are not. The difference between deletional HPFH and $\delta\beta^0$ thalassaemia is one of degree. The former has 15–30% haemoglobin F and almost balanced α and non- α chain synthesis whereas the latter has

Table 3.12 Inherited causes of a low haemoglobin A₂ (from references [369, 381, 382] and other sources).

δ^+ thalassaemia heterozygosity or homozygosity
δ^0 thalassaemia heterozygosity or homozygosity*
Very unstable δ chain variant
Some cases of $\delta\beta^0$ thalassaemia heterozygosity and all cases of $\delta\beta^0$ thalassaemia homozygosity* [369]
Some cases of $^A\gamma\delta\beta^0$ thalassaemia heterozygosity [369] and all cases of $^A\gamma\delta\beta^0$ thalassaemia homozygosity*
Deletional hereditary persistence of fetal haemoglobin heterozygosity and homozygosity* (except Vietnamese/South-East Asian type, which spares the δ gene and is associated with an increased haemoglobin A ₂)
α thalassaemia trait and haemoglobin H disease
Haemoglobin Lepore heterozygosity, compound heterozygosity† and homozygosity† (since one or both δ genes are lacking)
Haemoglobin Kenya heterozygosity
Heterozygosity for δ chain variants (but the total of A ₂ and variant A ₂ will generally be normal)
Trisomy D syndrome [381]
Bi-allelic null <i>KLF1</i> mutations [382]

* Absent haemoglobin A₂ in homozygotes (and compound heterozygotes with haemoglobin Lepore).

† Absent haemoglobin A₂ in homozygotes (and compound heterozygotes with $\delta\beta$ thalassaemia).

5–15% haemoglobin F and unbalanced chain synthesis. The distribution of haemoglobin tends to be pancellular in the former and hetero-cellular in the latter (Table 3.14). However, there is actually a continuous spectrum of disorders rather than two distinct groups and one condition that was previously designated type 6 HPFH [396, 397] is now considered to be more correctly characterised as $^A\gamma\delta\beta^0$ (i.e. $^C\gamma[^A\gamma\delta\beta]^0$) thalassaemia [369]. The molecular basis of the difference is not clearly understood; juxtaposition of a downstream enhancer to the γ genes in HPFH but not in $\delta\beta$ thalassaemia has been proposed as a mechanism [398].

The distribution of haemoglobin F between cells in various conditions associated with an increased percentage of haemoglobin F is best determined by flow cytometry.

A unique family has been reported in which HPFH (haemoglobin F 22% and 31%) was due to compound heterozygous mutation for a mis-sense and a nonsense mutation in a transcription factor gene, *KLF1*. There was also an increase in red cell protoporphyrin and a mild normocytic or microcytic haemolytic anaemia [387]. Haploinsufficiency of *KLF1* due to microdeletion can also be causative, with

values of 7% and 17% in two syndromic patients [399]. A very high haemoglobin F (37%) and the persistence of haemoglobin Portland has similarly been described in congenital dyserythropoietic anaemia type IV, associated with a *KLF1* mutation [400]. Compound heterozygosity for two *KLF1*-null mutations is associated with severe haemolytic anaemias with more than 70% haemoglobin F, persistence of haemoglobin Portland and low haemoglobin A₂ [382].

Deletional hereditary persistence of fetal haemoglobin

Deletional HPFH can result from a number of deletions of the β globin gene cluster, which are shown diagrammatically in Fig. 3.64. The haematological features of heterozygous subjects are summarised in Table 3.15 [369, 401–412] and blood film features are shown in Fig. 3.65. Deletional HPFH is quite common in some ethnic groups. Its prevalence in African-Americans is about 1 in 1000.

The first type of deletional HPFH to be recognised was an African type of $\delta\beta^0$ HPFH, described in an African-American child from

Table 3.13 Inherited conditions associated with high haemoglobin F percentage [250, 386–394].

Inherited abnormalities of globin genes

Heterozygotes and homozygotes for hereditary persistence of fetal haemoglobin

Deletional

Non-deletional

γ gene mutation

Mutation in the promoter of the $\text{G}\gamma$ or $\text{A}\gamma$ gene

β thalassaemia

Heterozygotes for β thalassaemia (some cases, particularly those with deletions of the 5' part of the β gene or promoter mutations)

Compound heterozygotes and homozygotes for β thalassaemia (β thalassaemia intermedia and major)

Heterozygotes and homozygotes for $\delta\beta$ and $\text{A}\gamma\delta\beta$ thalassaemia

Heterozygotes and homozygotes for haemoglobin Lepore

Heterozygotes for haemoglobin Kenya

Sickle cell trait

Some cases, particularly with the Saudi/Indian haplotype

Sickle cell anaemia and other forms of sickle cell disease

Some cases, particularly during treatment with hydroxycarbamide or with certain haplotypes: higher in Senegal haplotype than in Benin and Bantu haplotypes; particularly high in Saudi/Indian haplotype found in eastern province of Saudi Arabia and India

Unstable haemoglobins

Inherited abnormalities and polymorphisms other than those of globin genes

Genes controlling haemoglobin F synthesis

BCL11A haploinsufficiency due to microdeletion* or downregulation due to deletion of downstream regulatory element [386]

Heterozygous inactivating mutations of *KLF1* [250] and compound heterozygosity for *KLF1* mutations [387]

HBFQTL2 at 6q22.3-23.1, *HBS1L*–*MYB* intergenic region)

HBFQTL3 at Xp22.2

HBFQTL4 on 8q

Haematological disorders

Congenital aplastic anaemia (Fanconi anaemia)

Blackfan–Diamond syndrome, particularly during corticosteroid administration

Dyskeratosis congenita [388]

Congenital dyserythropoietic anaemia [389]

Shwachman–Diamond syndrome [390]

Hereditary spherocytosis [391]

Metabolic disorders

β -ketothiolase deficiency (high levels of butyric acid) [392]

Disorders of propionate metabolism [393]

Other

Osteopetrosis [394]

HBFQTL, haemoglobin F quantitative trait locus.

* There may be associated neurodevelopmental delay.

Baltimore. An alternative terminology is $(\delta\beta)^0$ or $\text{G}\gamma^{\text{A}\gamma}(\delta\beta)^0$ HPFH. There are now known to be at least six different deletions classified as $\delta\beta^0$ HPFH, two occurring in subjects of African descent (HPFH-1 and HPFH-2), one in Indians

(HPFH-3), two in Italians (HPFH-4 and HPFH-5) and one in Vietnamese/South-East Asians [369, 401, 402]. In the first five of these, both the δ and the β gene are deleted but the two γ genes are intact. Since homozygotes have no β or δ genes

Table 3.14 Distribution of haemoglobin F in various conditions associated with an increased haemoglobin F percentage.

Heterocellular	Pancellular
Some types of non-deletional HPFH	Some types of non-deletional HPFH
$\delta\beta$ thalassaemia	Deletional HPFH
Haemoglobin Lepore trait	Haemoglobin Kenya trait
Sickle cell anaemia, sickle cell/haemoglobin C disease and sickle cell/ β thalassaemia	Sickle cell/deletional HPFH compound heterozygosity
β thalassaemia heterozygosity and haemoglobin E/ β thalassaemia compound heterozygosity	β thalassaemia homozygosity and compound heterozygosity

HPFH, hereditary persistence of fetal haemoglobin.

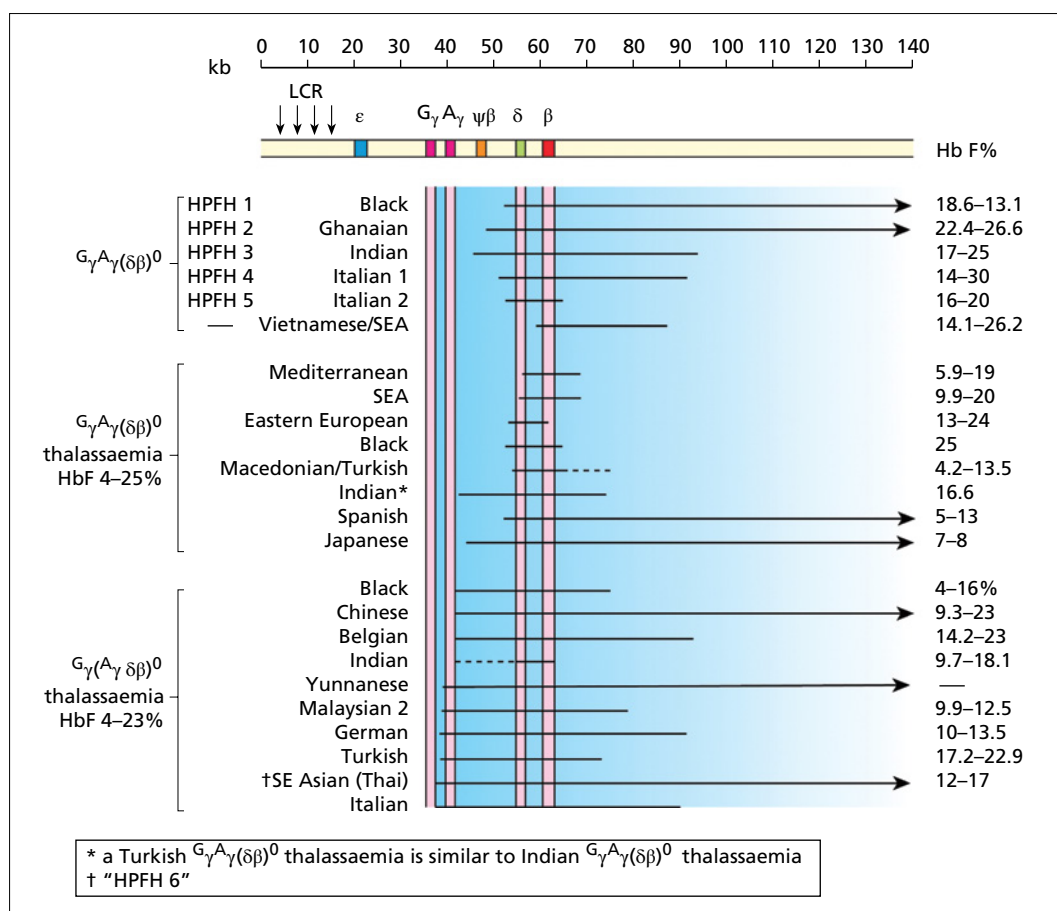


Fig. 3.64 Deletions resulting in hereditary persistence of fetal haemoglobin or in $\delta\beta$ thalassaemia (modified from reference [336]); $G_\gamma A_\gamma(\delta\beta)^0 = \delta\beta^0$ thalassaemia; $G_\gamma(A_\gamma\delta\beta)^0$ thalassaemia = $A_\gamma\delta\beta^0$ thalassaemia.

Table 3.15 Haematological features of heterozygosity for deletional hereditary persistence of fetal haemoglobin (HPFH) [369, 401–412].

Type of HPFH	Usual Hb F (%)	Usual Hb A ₂ (%)	Usual $\epsilon\gamma:\text{A}\gamma$ ratio	Molecular defect	Reference
African-American $\delta\beta^0$ (HPFH-1)	15–30*	1.2–2.7	50:50	Deletion including δ and β genes	[404, 411]
African (Ghanaian) $\delta\beta^0$ (HPFH-2)	20–30†	Reduced	30:70	Deletion including δ and β genes	[411]
Indian $\delta\beta^0$ (HPFH-3)	17–25	1.6–2.2	70:30	Deletion including δ and β genes	[405, 406]
Italian 1 (southern mainland Italy) $\delta\beta^0$ (HPFH-4)	14–30	1.7–2.0	35:65	Deletion including δ and β genes	[407]
Italian 2 (Sicilian) $\delta\beta^0$ (HPFH-5)	16,20	2, 2.1	15:85	Deletion including δ and β genes	[408]
Vietnamese/ South-East Asian	20.7 \pm 3.8	Increased (3.8 \pm 0.6)	60:40	Deletion of β gene	[369]
Haemoglobin Kenya	5–28‡	1.4–1.8	Mainly $\epsilon\gamma$	Deletion of part of the $\text{A}\gamma$ gene, all of the δ gene and part of the β gene with $\text{A}\gamma$ - β fusion	

* The percentage of haemoglobin F is very significantly reduced by coexisting iron deficiency [409].

† The percentage of haemoglobin F is very significantly reduced by coexisting iron deficiency [410].

‡ Plus 5–28% (usually 7–12%) of haemoglobin Kenya ($\alpha_2\text{A}\gamma\beta_2$) [412].

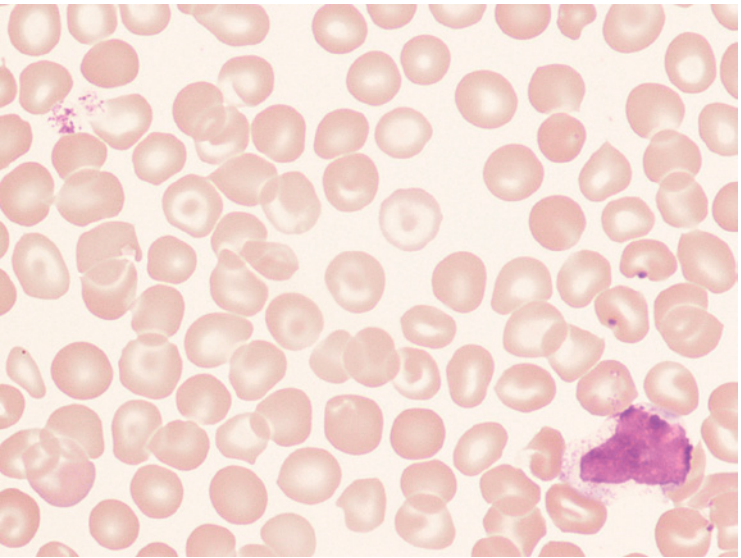


Fig. 3.65 Blood film of a patient who was heterozygous for HPFH showing target cells. MGG $\times 100$.

they cannot synthesise haemoglobin A or A₂. Haemoglobin F comprises 100% of haemoglobin. Both $\zeta\gamma$ and $\Lambda\gamma$ chains are synthesised but the proportion varies in the different subtypes. The synthesis of γ chain is almost sufficient to compensate for the lack of β chain synthesis so that there is no anaemia. In the Vietnamese/South-East Asian type the δ gene is retained.

Heterozygotes for $\delta\beta^0$ HPFH have a variable haemoglobin F percentage, depending on the deletion (see Table 3.15). The haemoglobin F percentage falls appreciably if a patient becomes iron deficient [410]. The haemoglobin A₂ percentage is either mildly reduced or normal, averaging around half of the normal mean level in most subtypes. The Hb is normal but the MCV and MCH may be somewhat reduced. The mean MCH varies from about 26pg in HPFH-1 to about 28pg in HPFH-3 [369]. The MCV shows similar variation between subtypes from a mean that is below the lower limit of normal to a mean that is clearly normal [369]. The blood film (Fig. 3.66) may be normal or show an occasional target cell. The morphological abnormalities are greater in homozygotes (Fig. 3.67). A Kleihauer test or flow cytometry shows pancellular distribution of haemoglobin F but there is some variation from cell to cell. The globin chain synthesis ratio is approximately normal.

Homozygotes are not anaemic. In fact, because haemoglobin F has a higher oxygen affinity than haemoglobin A, there may be mild polycythaemia. In some homozygotes the red cell indices resemble those of β thalassaemia trait with an increased RBC and reduced MCV and MCH. In others the RBC is towards the top and the MCV and MCH towards the bottom of their respective normal ranges. The reticulocyte count is normal. The blood film may show anisocytosis, poikilocytosis, mild hypochromia, mild microcytosis and target cells. 'Cells resembling spherocytes' (probably irregularly contracted cells), have been described [403]. Globin chain synthesis shows an imbalance similar to that in β thalassaemia trait with an α :non- α ratio of about 1.4–3.0. A Kleihauer test shows a pancellular distribution of haemoglobin F, an inevitable feature since only haemoglobin F is present.

Heterozygosity for haemoglobin Kenya (found in Kenyans and Ugandans) produces a variant of deletional, pancellular HPFH. Since one δ gene has been deleted there is a reduced proportion of haemoglobin A₂. Reported levels of haemoglobin Kenya have varied from 6% to 23% (mean 13%) and of haemoglobin F from 5% to 28% (mean 10%) [412]. Heterozygotes may be haematologically normal or show slight anaemia and occasional target cells. Globin chain synthesis is balanced.

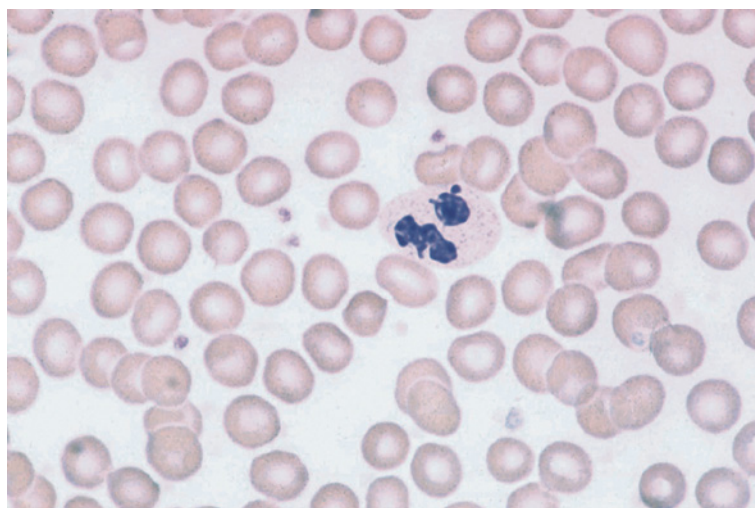


Fig. 3.66 Blood film from an adult African woman with hereditary persistence of fetal haemoglobin (HPFH). Red cell indices were RBC $4.2 \times 10^{12}/l$, Hb 120 g/l, MCV 88 fl, MCH 28.6, MCHC 324 g/l; there was 23% haemoglobin F and 1.6% haemoglobin A₂. MGG $\times 100$.

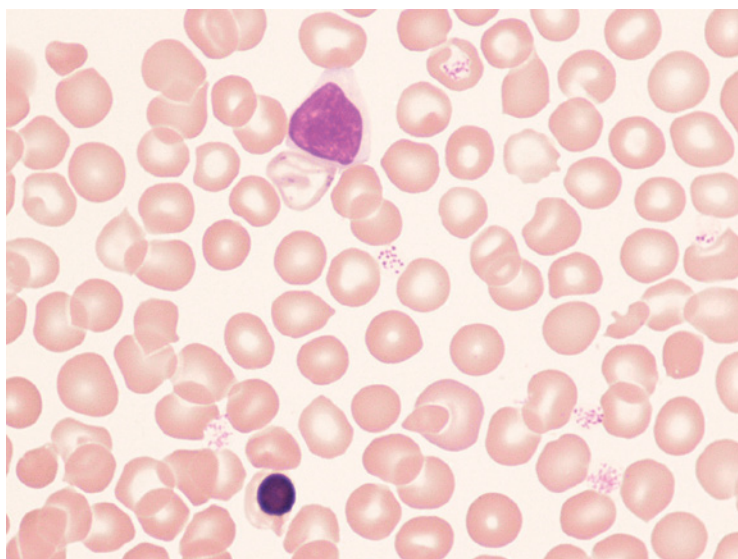


Fig. 3.67 Blood film of a 65-year-old West African male who was homozygous for Ghanaian HPFH showing hypochromia, microcytosis and target cells. There were also occasional nucleated red blood cells. Red cell indices were RBC $6.52 \times 10^9/l$, Hb 170 g/l and MCH 26.1 pg.

Interactions with other haemoglobinopathies

Deletional HPFH coinherited with β^s leads to a very mild sickling disorder with about 30% F and about 70% S [404, 413]. Haemoglobin A₂ may be low normal or decreased.

Compound heterozygotes for deletional HPFH and β thalassaemia have about 70% haemoglobin F, depending on the molecular basis of the HPFH and β thalassaemia alleles. The phenotype is variable. In the case of the two African types, the compound heterozygous state is phenotypically very mild whereas in the Indian type, although the heterozygotes do not have thalassaemic features, the compound heterozygotes have the clinical picture of thalassaemia intermedia [406, 413].

When coinherited with haemoglobin H disease in one family, HPFH was associated with some improvement in the Hb, a reduced proportion of haemoglobin H and 11% haemoglobin Bart's suggesting that the reduced number of α chains were demonstrating a greater affinity for β chains than for γ chains [414].

Non-deletional hereditary persistence of fetal haemoglobin

Non-deletional HPFH is a heterogeneous group of disorders. The most common form is associated with a polymorphism in regulatory

sequences of the β globin gene cluster. There is a C→T change at position -158 from the γ gene, which is readily detected because it creates a cleavage site for the enzyme Xmn1. For the sake of brevity, -158 γ C→T is used here to indicate this mutation and a similar notation is used for other mutations leading to non-deletional HPFH. A further polymorphism also influences the percentage of haemoglobin F, although it is not usually categorised with the HPFHs. It is based on repeat sequences within HS2 (hypersensitive site 2) of *LCRB*. The sequence is designated $(AT)_x N_{12}GT(AT)_y$ when X and Y are variable numbers of repeats of a sequence. There are at least eight different combinations of repeat sequences of which $(AT)_9 N_{12}GT(AT)_{10}$ is associated with an increased synthesis of haemoglobin F. Both C→T at -158 and $(AT)_9 N_{12}GT(AT)_{10}$ are associated with an increased number of F cells (and a small increase in haemoglobin F percentage). It has been suggested that the association -158 γ C→T with increased synthesis of haemoglobin F is consequent only on its linkage disequilibrium with $(AT)_9 N_{12}GT(AT)_{10}$, the latter polymorphism being much more strongly linked to an increased production of haemoglobin F in one study [415]. However, this seems unlikely in view of a considerable number of other studies that have linked -158 γ C→T to increased haemoglobin F in a variety of contexts.

Haemoglobin F concentration is also affected by genes encoding many different *trans*-acting factors. One is the determinant at Xp22.2, which influences F-cell production, and another has been mapped to 6q23.3 [182]. These give rise to increased synthesis of both $\zeta\gamma$ and $\Lambda\gamma$ globin chains and to $\zeta\gamma\Lambda\gamma$ HPFH.

Non-polymorphic non-deletional HPFH results mainly from point mutations (or occasionally a small deletion or insertion) involving the β globin gene cluster but not the δ and β globin genes themselves. The mutations are in and around highly conserved promoter motifs 5' to either the $\zeta\gamma$ or the $\Lambda\gamma$ gene. They are at -114, -117 or -175 from the transcription initiation sites of these genes or clustered around -158 to -161 or -195 to -202. These mutations are likely to alter the binding of *trans*-acting factors to the promoter. This type of non-deletional HPFH can be further categorised according to whether there is increased synthesis of $\zeta\gamma$ or $\Lambda\gamma$ chain. Increased synthesis of $\zeta\gamma$ chains results from mutation upstream of the $\zeta\gamma$ gene and

increased synthesis of $\Lambda\gamma$ chain from mutations upstream of the $\Lambda\gamma$ gene. Interestingly, the same mutations have often been observed in the same position 5' to one or other gene.

In non-deletional HPFH there is increased synthesis of haemoglobin F but haemoglobins A and A₂ continue to be synthesised, although at a reduced rate, so that α :non- α chain synthesis is fairly balanced. Whether the distribution of haemoglobin F is pancellular or heterocellular is partly a function of the proportion of haemoglobin F present and the sensitivity of the method used for its detection.

The reported types of non-deletional HPFH for which a molecular mechanism has been defined are shown in Table 3.16 [369, 384, 385, 401, 413–441]. It will be noted that, although the same mutation can occur 5' to either the $\zeta\gamma$ or the $\Lambda\gamma$ gene, the percentage of haemoglobin F characteristically seen may differ considerably.

Typical of pancellular $\zeta\gamma$ HPFH are the two point mutations at -202 and -175 from the $\zeta\gamma$ gene observed in subjects of African ancestry.

Table 3.16 Haematological features of non-deletional hereditary persistence of fetal haemoglobin (based on references [369, 384, 385, 401, 413–441]).

Type of HPFH	Ethnic group	Molecular defect	Percentage of haemoglobin F in heterozygotes	Percentage of haemoglobin F during erythropoietic stress	Reference
$\zeta\gamma$	African	-202 $\zeta\gamma$ C→G	15–25%*	19.9% and 23.5% with β^s in <i>trans</i>	[413]
	Tunisian	-200 $\zeta\gamma$ +C	18–27% (48 and 49% in homozygotes)		[369, 422]
	Black/ Sardinian/ British	-175 $\zeta\gamma$ T→C	17–30%*	29.5% with β^s in <i>trans</i>	[417–419]
	African	-161 $\zeta\gamma$ G→A	1–2%	More marked increase	[385, 441]
	Many ethnic groups, frequency of 0.32–0.35	-158 $\zeta\gamma$ C→T	2–3% or less (not always elevated if otherwise genetically normal)	More marked increase with β thalassaemia in <i>trans</i> ; 10% with β^s in <i>trans</i> ; 15% with $\beta^s\beta^s$	[420]
	African-American	-158 $\zeta\gamma\zeta\gamma$ C→T† (XmnI $\zeta\gamma$ polymorphism)	2.3–3.8%		[420]
	Australian	-114 $\zeta\gamma$ C→G	8.5%		[421]

(Continued on pp. 182–183.)

Table 3.16 *Continued.*

Type of HPFH	Ethnic group	Molecular defect	Percentage of haemoglobin F in heterozygotes	Percentage of haemoglobin F during erythropoietic stress	Reference
$\Lambda\gamma$	Japanese	-114 $G\gamma$ C→T	11–14%		
	Czechoslovakian	-110 $G\gamma$ A→C	1%		[441]
	Yugoslav	$G\gamma G\gamma \Lambda\gamma \ddagger$	About 5%		[423]
	Portuguese (1 family)	$G\gamma AG\gamma AG\gamma AG\gamma AG\gamma \Lambda\gamma$	0.3, 8%		[423]
	African	-202 $\Lambda\gamma$ C→T	1.6–3.9%	1.6–9% with S in <i>cis</i> ; 25% with SS	[385]
	British/Australian	-198 $\Lambda\gamma$ T→C	3.5–10%* (20% in homozygote)		
	Italian	-197 $\Lambda\gamma$ C→T	6%		[441]
	Italian/Chinese	-196 $\Lambda\gamma$ C→T [§]	12–21%*	38 and 40% with β thalassaemia in <i>trans</i> ; 20% with β^0 in <i>cis</i> ¶	[425, 426]
	Brazilian (Black, white or mixed)	-195 $\Lambda\gamma$ C→G	4.5–8.5%	7% with hereditary spherocytosis	[427]
	African-American	-175 $\Lambda\gamma$ T→C	17–38%	38% with β^C in <i>trans</i> and -158 $G\gamma$ C→T in <i>cis</i>	[425, 441]
	Cretan	-158 $\Lambda\gamma$ C→T**	Slight increase		[369]
	Greek/Sardinian/Black	-117 $\Lambda\gamma$ G→A	7–20%* (mean 13% in one series, 9.7% in another); 24% in two homozygotes and 37.6% in another; 13.5% in compound heterozygosity with -158 $G\gamma$ C→T	20–50% with β thalassaemia in <i>trans</i>	[429–435]
	African-American	-114 $\Lambda\gamma$ C→T	3–5%		[436]
	Italian	-113 $\Lambda\gamma$ A→G	6.5%		[441]
	Black	Del $\Lambda\gamma$ -114 to -102		30% and 31% with sickle cell trait	[437]
	Chinese	Del $\Lambda\gamma$ -226 to -223 (AAGC del)	5.4% in homozygote, 3.2% in heterozygote		[438]
	African	Del $\Lambda\gamma$ -225 to -222	6–7%		[441]

Table 3.16 *Continued.*

Type of HPFH	Ethnic group	Molecular defect	Percentage of haemoglobin F in heterozygotes	Percentage of haemoglobin F during erythropoietic stress	Reference
$\zeta\gamma^{\Delta\gamma}$	Many ethnic groups ('Swiss type')	Locus at Xp22.2-22.3	0.7–8.0%		
	Indian	Locus at 6q22.3-23.1, designated haemoglobin F quantitative trait locus 2 (<i>HBFQTL2</i>)	2.5–24%, cases with interaction with a β thalassaemia variant included		[439]
	British	Unknown autosomal locus other than 6q22.3-23.1	1–10.8% (interaction with –158 $\zeta\gamma$ C→T)		[384]
	Maltese††	<i>KLF1</i> at 19p13.12	3–19.5%		[440]

* Distribution of haemoglobin F is pancellular.

† Both γ genes are $\zeta\gamma$.

‡ However, triplication of the γ gene does not necessarily lead to increased haemoglobin F.

§ In Sardinia occurs with a β^0 mutation in *cis* producing the phenotype of $\delta\beta$ thalassaemia.

¶ So-called Sardinian $\delta\beta$ thalassaemia – actually this mutation plus β^0 thalassaemia.

** This mutation occurs in *cis* to –158 $\zeta\gamma$ C→T.

†† Associated hypochromia, microcytosis and poikilocytosis.

They show 15–25 and 17–30% respectively of haemoglobin F, almost all of $\zeta\gamma$ type. The compound heterozygous state with haemoglobin S indicates that the β gene in *cis* of the HPFH determinant continues to be expressed, albeit at a reduced rate. The second of these mutations has also been observed in Sardinians and white British individuals. Other $\zeta\gamma$ promoter mutations, leading to either heterocellular or pancellular HPFH, have been observed in blacks, Yugoslavians, white Australians and Japanese populations (see Table 3.14).

Typical of pancellular $\Delta\gamma$ HPFH is –117 $\Delta\gamma$ G→A, observed initially in Greeks but subsequently in Sardinians, Chinese and individuals of African ancestry. The percentage of haemoglobin F, of mainly $\Delta\gamma$ type, has generally been around 10–20%. Globin chain synthesis is balanced and there is no haematological abnormality. Two homozygotes have been described with approximately 75% haemoglobin A, 24% hae-

moglobin F and 0.8% haemoglobin A_2 , indicating that the δ and β genes in *cis* to the HPFH determinant are expressed, albeit at a reduced level.

Other $\Delta\gamma$ promoter mutations, leading to either pancellular or heterocellular HPFH, have been described in black, white (British, Australian, Italian) and Chinese individuals and in Brazilians of various ethnic origins (see Table 3.16). They generally result from point mutations but in one mutation, described in two individuals with sickle cell trait, there was a 13bp deletion involving the distal CCAAT box at –115 to –111 [438].

Coinheritance with other abnormalities of globin chain synthesis

Coinheritance of non-deletional HPFH ameliorates sickle cell anaemia and homozygous and heterozygous β thalassaemia. For example, in

both conditions, $-158 \text{ } ^6\gamma \text{ C} \rightarrow \text{T}$ and the $(\text{AT})_9\text{N}_{12}\text{GT}(\text{AT})_{10}$ polymorphism are associated with an increase in haemoglobin F synthesis. Homozygosity for $-158 \text{ } ^6\gamma \text{ C} \rightarrow \text{T}$ is also associated with an increased haemoglobin F percentage in haemoglobin E/ β thalassaemia and haemoglobin E disease [442].

A child with both $-117 \text{ } ^\alpha\gamma \text{ G} \rightarrow \text{A}$ non-deletional HPFH and the genotype of haemoglobin H disease has been reported [414]. The percentage of haemoglobin F was what was expected for the genotype, 9.5%, and haemoglobin A_2 was 1.3%. There was no haemoglobin H but haemoglobin Bart's was 11%, indicating that the reduced amount of α chain was combining preferentially with β chains rather than with γ chains.

The findings in other interactions of non-deletional HPFH and other haematological abnormalities are summarised in Table 3.16.

Other inherited abnormalities and haemoglobin F level

An increased proportion of haemoglobin F is observed in a variety of inherited conditions, related and unrelated to the β globin gene cluster (see Table 3.13).

A number of haemoglobinopathies are associated, in a proportion of cases, with an increased percentage of haemoglobin F. The increase of haemoglobin F can sometimes be linked to the nature of the β globin gene mutation. Heterozygotes for non-deletional β thalassaemia and 3' deletional β thalassaemia have been found to have a slight elevation of haemoglobin F (e.g. 1.5% in comparison with a normal of 0.7%) whereas haemoglobin Lepore heterozygotes had around 3% haemoglobin F and heterozygotes for 5' deletional β thalassaemia had around 3.5% [413]. Elevation of haemoglobin F in individuals with abnormalities of the β globin gene can also often be linked either to polymorphisms within the β globin gene cluster that affect binding of transcription factors or to other genetic factors. Heterozygotes for β thalassaemia or haemoglobin Lepore with a high F percentage have been found to have either the common $-158 \text{ } ^6\gamma \text{ C} \rightarrow \text{T}$ or $(\text{AT})_9\text{T}_5$ instead of $(\text{AT})_7\text{T}_7$ at -540 from the β gene [443]. Both of

these polymorphisms were found to exert an influence on haemoglobin F levels when found either in *cis* or in *trans* to the abnormal β or $\delta\beta$ globin gene. The $(\text{AT})_9\text{T}_5$ polymorphism was also linked to an increased percentage of haemoglobin F in β thalassaemia major [443].

The haemoglobin F level in sickle cell anaemia is determined by many factors (see page 246), including age, sex, possibly the X chromosome F-cell determining locus, various determinants in the β globin gene cluster and the number of α genes. However, it should be noted that interpretation of the F percentage in sickle cell disease, thalassaemia and some other haemoglobinopathies is complicated by the preferential survival of F-containing cells, both in the marrow and in the circulation.

In β thalassaemia major the haemoglobin F percentage is greatly elevated, in homozygotes for β^0 thalassaemia constituting almost all of the haemoglobin.

Inherited metabolic disorders can affect haemoglobin F synthesis when gene expression is altered by an abnormal metabolite, such as long-chain fatty acids in diabetes mellitus. Very abnormal haemopoiesis, for example in congenital dyserythropoietic or congenital aplastic anaemias, can also be associated with an elevation of haemoglobin F percentage. High haemoglobin F levels are also commonly seen in myelodysplastic syndromes and other clonal disorders of the marrow, and are characteristic of stress erythropoiesis, for example as occurs after sudden blood loss.

Check your knowledge

One to five answers may be correct. Answers to most questions can either be found in this chapter or can be deduced from information given.

- 3.1 An increased percentage of haemoglobin A_2 is expected in
 - (a) α thalassaemia trait
 - (b) β thalassaemia trait
 - (c) $\delta\beta$ thalassaemia trait
 - (d) $\gamma\delta\beta$ thalassaemia trait
 - (e) silent β thalassaemia trait

- 3.2 Genes forming part of the β gene cluster include
- (a) α
 - (b) β
 - (c) γ
 - (d) δ
 - (e) ϵ
- 3.3 Moderate to marked microcytosis is usually a feature of
- (a) haemoglobin H disease
 - (b) α^0 thalassaemia trait
 - (c) β thalassaemia trait
 - (d) heterozygosity for HPFH
 - (e) haemoglobin Lepore trait
- 3.4 Suitable methods for quantifying haemoglobin A₂ for the diagnosis of β thalassaemia trait include
- (a) inspection of an electrophoretic strip
 - (b) capillary electrophoresis
 - (c) cellulose acetate electrophoresis followed by densitometric scanning
 - (d) high performance liquid chromatography
 - (e) cellulose acetate electrophoresis followed by elution and spectrophotometry
- 3.5 Haemoglobin Bart's hydrops fetalis
- (a) is expected in about 50% of fetuses if both parents have α^0 thalassaemia
 - (b) is a likely outcome in West Africans if both parents have α thalassaemia trait
 - (c) is associated with an increased incidence of pregnancy-associated hypertension
 - (d) can cause developmental abnormalities in limbs
 - (e) is associated with good oxygen delivery to tissues
- 3.6 A decreased percentage of haemoglobin A₂ can be a feature of
- (a) α thalassaemia trait
 - (b) β thalassaemia trait
 - (c) haemoglobin Lepore trait
 - (d) iron deficiency anaemia
 - (e) $\delta\beta$ thalassaemia trait
- 3.7 α^0 thalassaemia
- (a) is common in African-Caribbeans
 - (b) in its homozygous form, leads to haemoglobin H disease
 - (c) is most often caused by deletion of both α genes on a single chromosome
 - (d) cannot usually be suspected from the red cell indices
 - (e) can be diagnosed by haemoglobin electrophoresis
- 3.8 HPFH can be caused by
- (a) deletions that include the β and δ genes
 - (b) deletion of an α gene
 - (c) deletion of a γ gene
 - (d) mutation in a gene on chromosome 16
 - (e) point mutations upstream of either the γ or the γ^A globin gene
- 3.9 $\delta\beta$ thalassaemia
- (a) leads to an increased percentage of haemoglobin F
 - (b) leads to an increased percentage of haemoglobin A₂
 - (c) usually results from deletion of the δ and β genes
 - (d) when homozygous may lead to the phenotype of thalassaemia intermedia
 - (e) may be simulated by the coinheritance of δ and β thalassaemia
- 3.10 Haemoglobin Bart's hydrops fetalis
- (a) usually results from homozygosity for α^+ thalassaemia
 - (b) is mainly seen in those of Chinese or South-East Asian origin
 - (c) is characterised by worse tissue hypoxia than would be predicted from the haemoglobin concentration
 - (d) is characterised by low serum albumin and generalised oedema
 - (e) occurs occasionally in Greeks and Cypriots

- 3.11 The phenotype associated with β thalassaemia homozygosity or compound heterozygosity may be ameliorated by
- coinheritance of $\alpha\alpha$ (triple alpha)
 - coinheritance of γ thalassaemia
 - coinheritance of non-deletional HPFH
 - coinheritance of α^0 thalassaemia heterozygosity
 - coinheritance of α^+ thalassaemia homozygosity
- 3.12 The following variant haemoglobins lead to an α or β thalassaemia phenotype
- haemoglobin S
 - haemoglobin C
 - haemoglobin E
 - haemoglobin Lepore
 - haemoglobin Constant Spring
- 3.13 β thalassaemia trait with a normal haemoglobin A₂ percentage may be caused by
- coexisting δ thalassaemia
 - dominant β thalassaemia
 - a β thalassaemia mutation association with only a minor reduction of β chain synthesis
 - coinherited triple α
 - β thalassaemia caused by deletion of LCRB

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Answers to questions

- | | | | |
|---|---|--|--|
| 3.1 (a) F
(b) T
(c) F
(d) F
(e) F | 3.5 (a) F
(b) F
(c) T
(d) T
(e) F | 3.9 (a) T
(b) F
(c) T
(d) T
(e) T | 3.13 (a) T
(b) F
(c) T
(d) F
(e) T |
| 3.2 (a) F
(b) T
(c) T
(d) T
(e) T | 3.6 (a) T
(b) F
(c) T
(d) T
(e) T | 3.10 (a) F
(b) T
(c) T
(d) T
(e) T | |
| 3.3 (a) T
(b) T
(c) T
(d) F
(e) T | 3.7 (a) F
(b) F
(c) T
(d) F
(e) F | 3.11 (a) F
(b) F
(c) T
(d) T
(e) T | |
| 3.4 (a) F
(b) T
(c) F
(d) T
(e) T | 3.8 (a) T
(b) F
(c) F
(d) F
(e) T | 3.12 (a) F
(b) F
(c) T
(d) T
(e) T | |

4 Sick cell haemoglobin and its interactions with other variant haemoglobins and with thalassaemias

The first description of sickle cell disease is generally attributed to James Herrick who, in 1910, reported that a patient with severe anaemia had 'peculiar elongated and sickle shaped red blood corpuscles' – an observation that had been initially made by his intern, Ernest Lyons [1]. The term 'sickle cell anaemia' was first used by Verne Mason in 1922 [2]. Several decades later Linus Pauling and colleagues found that the sickling phenomenon was caused by haemoglobin with unusual characteristics [3] and subsequently Vernon Ingram and colleagues identified the causative amino acid change in the β chain of haemoglobin [4]. Sick cell haemoglobin, haemoglobin S, has a valine for glutamic acid substitution at the sixth amino acid of the β globin chain. The haemoglobin can be designated $\alpha_2\beta_2^{7\text{ Glu}\rightarrow\text{Val}}$, and the change in the β globin chain designated (HBB; c.20A>T, p. Glu7Val). Sick cell haemoglobin can produce deleterious effects because, on deoxygenation, its solubility is reduced and polymerisation occurs (Fig. 4.1). Both partially and fully deoxygenated haemoglobin S can be incorporated into a polymer. Long polymers distort the red cell into a holly-leaf, crescent or sickle shape that hinders blood flow through capillaries, both because of reduced deformability and because of increased adhesion to endothelial cells resulting from secondary changes in the red cell membrane. The lag phase before polymerisation occurs is determined by the partial pressure of O_2 , temperature, pH and haemoglobin S concentration within the red cell. When fully oxygenated, haemoglobin S is as soluble as haemoglobin A. Although haemoglobin A can copolymerise with haemoglobin S, the presence of haemoglobin A

in a red cell slows polymerisation because the concentration of haemoglobin S is less. Haemoglobin F and haemoglobin A₂ are even more effective at retarding polymerisation since they do not copolymerise with haemoglobin S and the hybrid tetramer, $\alpha_2\beta^s\gamma$, is similarly unable to polymerise. In comparison with haemoglobin A, polymerisation is facilitated by the presence of haemoglobin C, haemoglobin D-Punjab/D-Los Angeles or haemoglobin O-Arab. Because acidosis and a rise in temperature shift the oxygen dissociation curve to the right, they favour polymerisation. However, in clinical practice, exposure to cold can provoke sickling because of slowed circulation through capillaries and therefore increased periods of deoxygenation.

The sickle cell mutation occurs on several different genetic backgrounds (haplotypes) which has led to speculation that it may have occurred on several separate occasions. More recent studies, using whole-genome-sequence data, showed that there was a common ancestral haplotype, suggesting that the sickle mutation arose on a single occasion 7300 years ago, and subsequently moved to different haplotypes by gene conversion (Fig. 4.2) [5]. There are three main foci of haemoglobin S in Africa, associated with different haplotypes, the haplotypes being defined by restriction fragment length polymorphism (RFLP) analysis. They are in Senegal (Senegal type), the Central African Republic and southern Africa (Bantu or Central African Republic type) and Benin, Central, West and North Africa (Benin type) [6]. The Benin type has also spread to Spain, Portugal, Sicily (perhaps from Greece, perhaps from Sudanese soldiers in Arab armies) and southern mainland

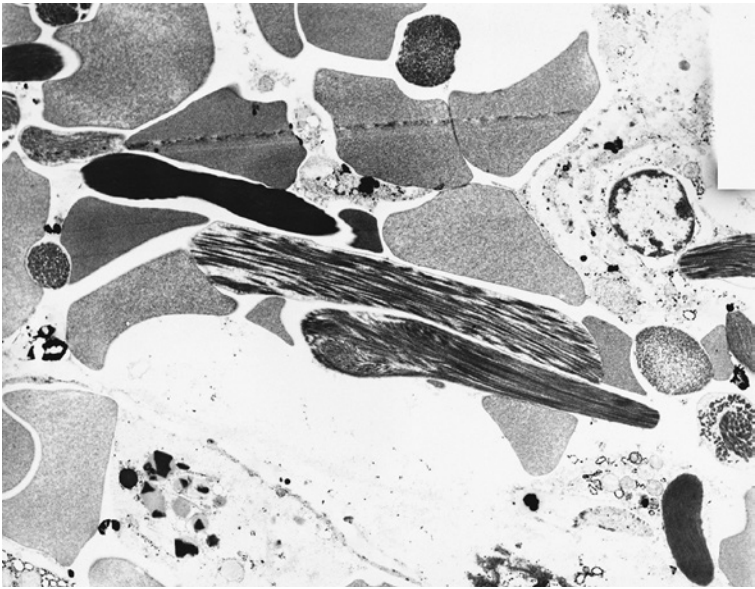


Fig. 4.1 Transmission electron micrograph showing polymerisation of haemoglobin S in a patient with compound heterozygosity for haemoglobin S and haemoglobin D-Punjab. (With thanks to Mr S. Ladva.)

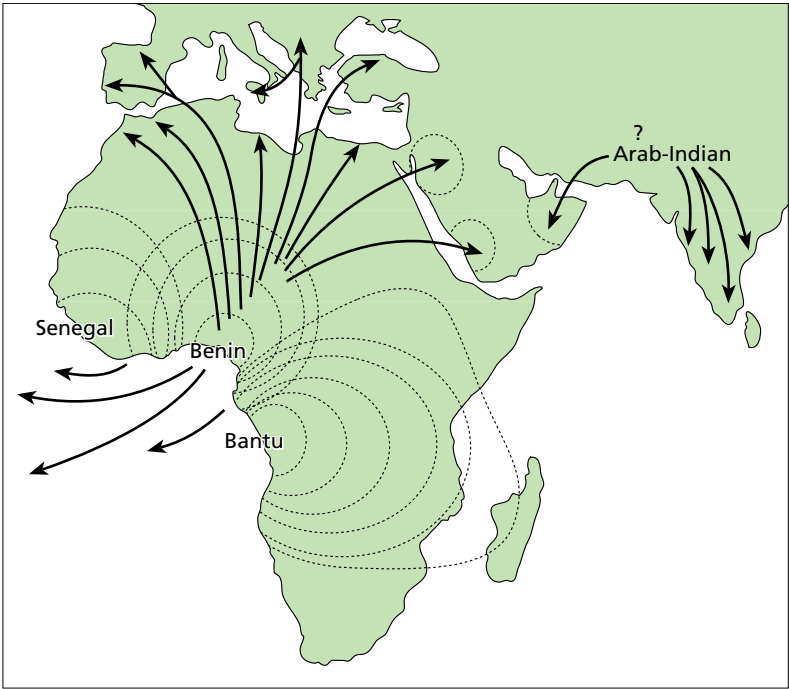


Fig. 4.2 The multifocal origin and spread of the β^S gene.

Italy, Greece (particularly Macedonia), Albania, Turkey, northern and south-western Saudi Arabia, Yemen and Oman. The Bantu type has spread to Kenya, Zambia and the Sudan. A different haplotype is associated with further foci in eastern Saudi Arabia, Kuwait, Bahrain,

Iran and Oman and in extensive areas of central and southern India, particularly among the scheduled tribes (a group living outside the caste system). It is designated the Arab-Indian haplotype. The prevalence of the haemoglobin S gene is up to 25% in eastern Saudi Arabia and

as high as 30% in some tribal populations in central India. The Indian/Saudi Arabian haplotype has also been found in Afghanistan and among Bedouin Arabs in Israel.

Migration, mostly from African countries, has led to the sickle cell variant occurring throughout the world, particular in North and South America, and most of Europe. For example, the prevalence of the β^S allele at birth in England has been estimated at 0.39 per 1000 [7] and in parts of Belgium (Brussels and Liege) at 15 per 1000 [8]. There is a high prevalence in some populations in Mexico, Colombia, Venezuela, Guyana, Suriname, French Guiana, Brazil and Peru. All three major African haplotypes are represented in the USA, the Caribbean and the UK. The sickle cell allele is also found in Madagascar, Mauritius (both Bantu and Arab-Indian haplotypes), Abu Dhabi, United Arab Emirates, Lebanon, Iraq, the southern part of the previous USSR and among North African Arabs.

The wide geographic spread of a potentially deleterious gene has been attributed to protection of heterozygotes from premature death from falciparum malaria. In areas where malaria is endemic, the β^S and β^A alleles may exist as balanced polymorphism (i.e. death or serious disability from sickle cell anaemia before the age of reproduction is balanced by a decreased death rate from malaria among heterozygotes). The prevalence of haemoglobin S in various populations is shown in Table 4.1 [9–41]. The highest birth incidence of sickle cell disease (not just sickle cell anaemia) is found in Equatorial Guinea, Benin, Burkina Faso, Nigeria, Sierra Leone and Togo, all of which have a birth incidence of more than 20 per 1000 live births [42]. A birth incidence of between one in 1000 and one in 2000 is found in Bahrain, Angola, Democratic Republic of the Congo, Kenya, Ghana, Guinea, Niger and São Tomé and Príncipe [42].

A second mutation is occasionally present in a gene that carries the β^S variant leading to synthesis of a variant haemoglobin with two amino acid substitutions. This could arise either through a second mutation occurring in a gene that already has a mutation, or through

crossover between genes encoding two different variant haemoglobins; for example, haemoglobin C-Harlem could have arisen through crossover between β^S and $\beta^{Korle Bu}$. Such variant haemoglobins retain their ability to sickle and in heterozygous, homozygous and compound heterozygous states can cause sickle cell disease of varying severity. At least 16 such double mutations are known (Table 4.2) [13, 43–50]. The most common, haemoglobin C-Harlem (initially described under the name of haemoglobin C-Georgetown), $\alpha_2\beta_2^{6Glu \rightarrow Val, 73Asp \rightarrow Asn}$, is less prone to polymerisation than haemoglobin S itself. The rare double substitution haemoglobin, haemoglobin S-Antilles, is more prone to polymerise than haemoglobin S itself, as are haemoglobins Jamaica Plain and S-Oman. Some double substitution haemoglobins differ from haemoglobin S in their mobility on cellulose acetate electrophoresis and capillary electrophoresis, and in their retention time on high performance liquid chromatography (HPLC).

There are other very rare haemoglobins unrelated to haemoglobin S that can polymerise *in vitro* and cause pseudosickling of red cells, including haemoglobin I ($\alpha 16 \text{ Lys} \rightarrow \text{Glu}$) and haemoglobin Setif ($\alpha 22 \text{ Asp} \rightarrow \text{Tyr}$). Although they are not associated with any relevant clinical abnormality, haemoglobin Setif can cause a false positive sickle solubility test [51]. There are also other haemoglobins that have the same amino acid substitution at a different site and which have the same characteristics as haemoglobin S on HPLC but which do not lead to sickling; for example, haemoglobin Haaglanden has the same characteristics on HPLC although mobility is slightly different on capillary electrophoresis [52]; a sickle solubility or equivalent test is important in making the distinction.

Homozygosity for haemoglobin S ($\beta^S\beta^S$) causes a serious condition referred to as sickle cell anaemia. Heterozygosity for haemoglobin S ($\beta^S\beta$), referred to as sickle cell trait or being a sickle cell carrier, is largely asymptomatic. The β^S gene may also be coinherited with another β chain variant. When there is deleterious interaction between the sickle cell haemoglobin

Table 4.1 The prevalence (%) of haemoglobins S and C in different populations. (From references [9–41] and other sources.)

Country or people	Variant haemoglobin	
	Haemoglobin S	Haemoglobin C
West Africa		
Senegal	3–15	<1–6
Gambia	6–28	<1–2
Guinea Bissau	<1–25	<1–1.5
Guinea	13–33	
Sierra Leone	22–30	
Liberia	<1–29 (10% in Monrovia, 1% SS at birth) [37]	1–3 (<1% in Monrovia) [37]
Ivory Coast	2–26	<1–50
Mali	5–17	
Burkina Faso	2–34 1.75% sickle cell disease (SC and SS) at birth	15–40
Ghana	3–25 (2% SCD)	8–40
Togo	6–28	7–17
Benin	5–31 (30% of pregnant women AS, SC or SS)	7–27 (8% of pregnant women AC, SC or CC)
Niger	5–23	1–8
Nigeria	10–41 (1–2% birth prevalence of sickle cell anaemia)	<1–9 (0.24% SC)
Central Africa		
Gabon	8–32	
Cameroon	<1–31	<1
Central African Republic	2–24	
Republic of the Congo (Congo-Brazzaville)	7–32	
Democratic Republic of the Congo (formerly Zaire)	1–46 (17% AS, 1.4% SS at birth)	
East Africa		
Kenya	<1–34	
Uganda	1–39 (nationwide 13.3%) [35]	
Tanzania	1–38 (overall 13%) [36], 21% birth prevalence in north west [41]	
Rwanda		
Tutsi	<1–5	
Hutu	5–15	
Burundi	1.5–26	
Southern Africa		
Angola	8–40	<0.1%
Zambia	<1–30	
Zimbabwe	<1–11	
Malawi	3–18	
Mozambique	<1–40	
Madagascar	<1–23 (7% on coast, 1.5% in highlands)	
Botswana	<1	
Namibia	0–15	

Table 4.1 *Continued.*

Country or people	Variant haemoglobin	
	Haemoglobin S	Haemoglobin C
South Africa		
Bantu	<1–4	
Indian	2–10	
Cape Coloured	<1	<1
North Africa		
Morocco	<1–7	<1–6
Algeria	<1–15	<1–13
Tunisia	<1–2	
Libya	<1–6	
Egypt	<1*	
Sudan	<1–17	≈ 0.4%
Horn of Africa		
Ethiopia	0–1	
Djibouti	≈ 0	
Somalia	≈ 0	
USA		
African Americans	7–8%	1–3.5
US Hispanic	0.7%	
US residents overall	1.6%	
African Caribbeans		
Jamaica	3.5–12 (0.6% SCD)	2–4
Bahamas	14	3
Barbados	4	3–5
Cuba	0–23	0–2.5
Haiti	7–17	1–3
Dominican Republic	6–12	3
Puerto Rico	<1–8	<1–2
Lesser Antilles	1–14	1–4.5
Guadeloupe	0.2–4.4	
Martinique	8	
Curacao	2	
Central America		
Mexico	<1–9	<1
Guatemala	<1–17	
Belize	0–25	
El Salvador	<1–2	
Honduras	<1–16	
Nicaragua	≈ 0	
Costa Rica	<1–8	
Panama	0–21	0–2.5
South America		
Colombia	0–15	0–6
Venezuela	0–9	0–3
Guyana	<1	
Suriname	0–22	0–6
French Guiana	0–18	0–7
Ecuador	≈ 0	≈ 0

(Continued on pp. 213–214.)

Table 4.1 *Continued.*

Country or people	Variant haemoglobin	
	Haemoglobin S	Haemoglobin C
Peru	<1	≈0
Bolivia	≈0	≈0
Brazil	0–16	0–4
Paraguay	≈0	
Argentina	<1	
Uruguay	≈0	
Chile	<1	<1
Greece	0–32	
Turkey	<1–34	0.5–1
Cyprus	<1	
Italy		
Sicily	<1–13%	0.6%
Sardinia	≈0	
Mainland southern	0.5–1	
Portugal	<1–5	
Spain	0.1 (indigenous Spaniards); SCD 3/100 000 births [38]	0.12 (southern Spain)
Eastern Europe		
Albania	3.2% (one study)	
Middle East		
Turkey	<1–34	
Syria	<5–25	
Lebanon	<1 (about 0.1% SCD)	
Jordan	4–6	
Israel†		
Arabs	1–38	
Jews	≈0	
Iraq	0–25	
Iran	≈0.03	
Saudi Arabia	<1–36 (overall 4.2% heterozygosity, 0.25% SCD)	
Kuwait	2	
Bahrain	11–16	
Oman	5	Rare
Yemen	1–2‡	
Abu Dhabi	2	
United Arab Emirates	2–5%	
Qatar	7.5%	
Asia		
India	0–35§	
Pakistan	0.5–1	
Sri Lanka	0–1.05 in different districts [39]	Very rare
Thailand		Rare
Nepal	≈ 9% in the Tharu population [40]	

AC, haemoglobin C trait; AS, sickle cell trait; CC, homozygosity for haemoglobin C; SC, compound heterozygosity for haemoglobins S and C; SCD, sickle cell disease; SS, sickle cell anaemia.

* 5–22% in various oases.

† Highest in Bedouin tribes.

‡ 23% in Western province.

§ 5–35% in various tribal populations [21]; 15% in Orissa, Madhya Pradesh and Maharashtra states [22].

Table 4.2 Variant haemoglobins in which haemoglobin S is one of two mutations [13, 43–50].

Variant haemoglobin	Second substitution	Mobility on cellulose acetate at alkaline pH
C-Harlem*	$\beta 73 \text{ Asp} \rightarrow \text{Asn}$	C
C-Ziguinchor	$\beta 58 \text{ Pro} \rightarrow \text{Arg}$	C
S-Travis	$\beta 142 \text{ Ala} \rightarrow \text{Val}$	S
S-Antilles†	$\beta 23 \text{ Val} \rightarrow \text{Ile}$	S [43]
S-Providence	$\beta 82 \text{ Lys} \rightarrow \text{Asn}$	A
S-Omant†	$\beta 121 \text{ Glu} \rightarrow \text{Lys}$	With C or slower than C
S-Wake*	$\beta 139 \text{ Asp} \rightarrow \text{Ser}$	[44]
S-Cameroon	$\beta 90 \text{ Glu} \rightarrow \text{Lys}$	A ₂
Jamaica Plain†	$\beta 68 \text{ Leu} \rightarrow \text{Phe}$	S [45]
S-South End	$\beta 132 \text{ Lys} \rightarrow \text{Asn}$	A [46]
C-Ndjamena*	$\beta 37 \text{ Trp} \rightarrow \text{Gly}$	C
S-Clichy	$\beta 8 \text{ Lys} \rightarrow \text{Thr}$	Between A and F [47]
S-San Martin‡	$\beta 85 \text{ Phe} \rightarrow \text{Leu}$	S [48]
C-New Cross	$\beta 83 \text{ Gly} \rightarrow \text{Asp}$	Slightly slower than S (between S and C on acid agarose) [49]
S-São Paulo§	$\beta 65 \text{ Lys} \rightarrow \text{Glu}$	Faster than A [50]
S-Northwick	$\beta 37 \text{ Trp} \rightarrow \text{Gly}$	E zone on capillary electrophoresis

* Similar disease to sickle cell anaemia in compound heterozygote.

† Sickling can occur in heterozygotes and S-Antilles/C compound heterozygotes; more severe disease than sickle cell anaemia occurs in compound heterozygote with haemoglobin S.

‡ Unstable, chronic haemolytic anaemia with exacerbations in two heterozygotes, microcytosis, variant haemoglobin 16 and 18%.

§ Mildly unstable, moderate anaemia in a heterozygote.

and the second variant haemoglobin, as is the case, for example, with haemoglobin C and haemoglobin D-Punjab, a clinically significant sickling disorder occurs. Subjects who are heterozygous for β thalassaemia and haemoglobin S likewise suffer from the clinicopathological effects of sickle cell formation and consequent complications. The term 'sickle cell disease' is sometimes used as a synonym for sickle cell anaemia and sometimes as a generic term to include sickle cell anaemia and other conditions in which a clinically significant disorder results from sickle cell formation and the associated pathological processes. Usage of the term 'sickle cell disease' to refer to sickle cell anaemia is not recommended since this can cause confusion. To avoid ambiguity, the term

should be defined whenever used. Some significant compound heterozygous states are shown in Table 4.3 [43–45, 53–56].

Sickle cell trait

The term 'sickle cell trait' indicates heterozygosity for the sickle cell allele ($\beta\beta^S$). Sickle cell trait does not generally cause haemolytic or vaso-occlusive complications, and is asymptomatic in the great majority of individuals, but is of genetic importance. It gives partial protection against uncomplicated malaria, cerebral malaria, severe malaria and death from *Plasmodium falciparum* malaria; in Africa, it is also associated with protection against neonatal illness, severe malnutrition and bacteraemia, probably because of the protection against

Table 4.3 Causes of sickle cell disease [43, 45, 53–56].

Sickle cell anaemia (homozygosity for haemoglobin S)

Compound heterozygous states

Sickle cell/haemoglobin C disease
Sickle cell/ β thalassaemia
Sickle cell/haemoglobin D-Punjab
Sickle cell/haemoglobin C-Harlem
Sickle cell/haemoglobin S-Antilles
Sickle cell/haemoglobin O-Arab
Sickle cell/haemoglobin Quebec-Chori [53]
Sickle cell/haemoglobin S-Oman
Sickle cell/haemoglobin O-Tibesti
Sickle cell/haemoglobin Monroe
Haemoglobin S-Antilles /haemoglobin C
Sickle cell/haemoglobin Lepore
Haemoglobin C/haemoglobin C-Harlem [54]

Mutations leading to sickle cell disease in heterozygotes (dominant sickle cell disease)

Haemoglobin S-Antilles [43]
Haemoglobin S trait plus haemoglobin Conakry trait (an α chain variant) [55]
Haemoglobin S-Oman [56]
Haemoglobin Jamaica Plain [45]

malaria [57]. However, the incidence of asymptomatic malaria is not decreased [58] and this protection is lost if there is coexistence of α thalassaemia heterozygosity [59].

If a patient with symptoms suggestive of sickle cell disease appears to have sickle cell trait on haemoglobin electrophoresis or HPLC, further detailed investigation is indicated since this can be the result of a second mutation in a β^S gene, such as haemoglobin Jamaica Plain (see later), or a second mutation in a β^C gene, such as haemoglobin Arlington Park (see later). The second mutation alters the characteristics of the variant haemoglobin so that it can be confused with haemoglobin A.

Sickle cell trait is sometimes an opportunistic diagnosis following detection of a variant haemoglobin during measurement of haemoglobin A_{1c} in patients with diabetes mellitus. Confirmation of the suspected abnormality without specific consent has been found to be acceptable to the great majority of patients [60]. Conversely, an incidental diagnosis of diabetes mellitus may be made in a

patient being investigated for sickle cell trait. It should also be noted that underestimation of prior glycaemia by haemoglobin A_{1c} measurement has been reported in patients with sickle cell trait, but analytical interference rather than a biological difference has not been excluded [61].

Clinical features

People with sickle cell trait are usually asymptomatic. However, sickle cell formation leading to vaso-occlusion can occur during high fever and under conditions of significant hypoxia such as during travel by air (particularly but not only in unpressurised aircraft), during mountain climbing, ascending in a cable car and skiing at altitude, during vigorous exercise and during anaesthetic misadventures. Five percent of cells may be sickled during high altitude flying [62]. Vaso-occlusion in such circumstances can lead to splenic, pulmonary, pituitary, cerebral, retinal, renal and bone infarcts and also to priapism (persistent erection caused by sickling within blood vessels of the penis). Bone infarction can lead to avascular necrosis. There have been more than 50 reports of splenic infarction in individuals with sickle cell trait related to altitude [63, 64]; this can be followed by abscess formation [64]. Exceptionally, splenic infarction necessitating splenectomy was reported in a father and son who ascended by road to 7200 feet [65]. Hypoxia caused by pneumonia [66] or pulmonary embolism [67] has also been responsible. Splenic infarction has been precipitated by Epstein-Barr virus infection [68, 69] and by exposure to cold [70]. Splenic infarction has been reported in a number of patients with coexisting hereditary spherocytosis, being attributed to sickling induced by hypoxia due to slow passage of spherocytes through the spleen [71]. Splenic infarction was also been reported in one patient with coexisting sickle cell trait and severe pyruvate kinase deficiency [55]. Spontaneous splenic rupture has been reported [72], in one instance in association with bacterial endocarditis [73]. Splenic sequestration has likewise been described, but very rarely, in

sickle cell trait [74]. Stuttering priapism unrelated to altitude can occur and can respond to exchange transfusion if more conservative measures fail [75]. There is a small increased risk of sudden death associated with vigorous exercise, particularly exercise at a high altitude and exercise complicated by dehydration and acidosis [76]. Such circumstances can also lead to exertional rhabdomyolysis, disseminated intravascular coagulation and renal failure [77]. Rhabdomyolysis and acute compartment syndrome have also been reported following vigorous exercise in the absence of complicating factors such as a high environmental temperature or dehydration [78]. In a study of US Air Force personnel the rate of non-traumatic deaths in airmen was very low but was 25-fold higher in those with sickle trait than in those without sickle trait [79]. Similar observations have been made in US army recruits. Because of the possibility of exertional sickling, the US National Athletic Trainers Association recommended that colleges and universities test student-athletes for sickle cell trait. However, the US Armed Forces and the American Society for Hematology prefer a policy of risk reduction in all military personnel and athletes [80]; with this policy being followed in US soldiers, the risk of exertional rhabdomyolysis is higher in those with sickle cell trait but there is no significant increase in the risk of death [81]. Sickle cell trait has been related to a higher rate of vascular complication in Africans with diabetes mellitus [82]. Sickle cell trait was associated with intraflap thrombosis and sickling in a patient having breast reconstruction, cooling of the flap being suspected as a precipitating factor [83]. Rarely proliferative retinopathy has been reported [84].

In sickle cell trait, spontaneous sickle cell formation can occur in renal papillae where oxygen tension is normally low, leading to renal papillary necrosis, episodes of haematuria [85] and impairment of renal concentrating ability (hyposthenuria), which can be associated with enuresis in children. Loss of renal concentrating ability is less if α thalassaemia trait coexists [86]. In African Americans, the estimated glomerular filtration rate is lower in individuals with sickle

cell trait than in those without [87]. There is predisposition to proteinuria [85] and an increased risk of chronic kidney disease [85, 88–91] with end stage renal disease being about twice as common as in Caucasians [90]. There is a statistically significant increased risk of ischaemic stroke in those with chronic kidney disease but not in those without [91]. In African American US army soldiers, sickle cell trait is associated with a higher incidence of acute kidney injury as well as chronic kidney disease [92]. Coinheritance of α thalassaemia protects against chronic kidney disease [93]. There is also a quite strong association between sickle cell trait and medullary carcinoma of the kidney [94, 95], this rare carcinoma having also been reported in sickle cell anaemia and sickle cell/haemoglobin C disease [96]. Sickled cells have been observed in the urine in a patient with sickle cell trait being investigated for post-partum fever [97].

During pregnancy, women with sickle cell trait may have an increased incidence of bacteriuria and pyelonephritis [98] and pregnancy-associated hypertension [99]. Other pregnancy-associated complications include a more than doubled incidence of post-partum endometritis and a statistically, but probably not clinically, significant decrease in the period of gestation at delivery and the birth weight [99]. Sickle cell trait has been associated with a higher rate of stillbirth [100].

If the kidney is excluded, spontaneous episodes of vascular occlusion (i.e. episodes occurring in the absence of fever, dehydration, hypoxia or acidosis) are very rare but do occur. One instance of a cerebrovascular accident associated with moya moya has been reported [101]. In one study the overall risk of coronary artery disease, congestive heart failure and stroke was found *not* to be increased in a predominantly young population [88] but another long term prospective study found a significantly higher risk of ischaemic stroke [102].

In one study the incidence of deep vein thrombosis was increased about twofold and the incidence of pulmonary embolism about fourfold [103]. In another study the incidence of pulmonary embolism was increased 1.37-fold [88].

D-dimer concentration is higher in African Americans with sickle trait than those without [87].

An increased incidence of invasive pneumococcal disease has been reported [104].

Because of the low pH of the anterior chamber of the eye, post-traumatic hyphaemia can lead to local sickling, impaired outflow and increased intraocular pressure necessitating surgical evacuation. Screening for sickle cell trait is therefore indicated in any person of an appropriate ethnic group presenting with a hyphaemia. Proliferative retinopathy has been reported in individuals with coexisting ocular or systemic disease [105] but not otherwise.

In one study sickle cell trait caused a small but significant increased risk of hospitalisation and death from SARS-CoV-2 (severe acute respiratory syndrome-coronavirus-2) infection [106] while in another sickle cell trait was more prevalent in African Americans hospitalised with SARS-CoV-2 infection than in the community but morbidity and mortality were not increased [107]. However, a large study of US veterans found sickle cell trait to be associated both with higher mortality from COVID-19 (coronavirus disease 2019) and a higher incidence of COVID-associated acute kidney injury [108].

An extensive epidemiological study found individuals with sickle cell trait to have a

statistically significant increase not only in renal disease (renal papillary necrosis, chronic kidney disease and end stage renal failure) but also in type 2 diabetes, hypertension, retinopathy and rhabdomyolysis [109].

Despite this list of potential complications the great majority of patients with sickle cell trait are asymptomatic and the main reason for seeking to identify the heterozygous state is the genetic implications. If both parents have sickle cell trait there is a 25% probability of sickle cell anaemia in a child.

Laboratory features

Blood count

The haemoglobin concentration (Hb) is normal except in those with coexisting α thalassaemia trait who may be slightly anaemic [110, 111]. The mean cell volume (MCV) and mean cell haemoglobin (MCH) are reduced in those with coexisting α thalassaemia trait (Table 4.4) [13, 38, 110–115]. However, in a large survey carried out in Kenyan children, MCV and MCH were also reduced and the red cell count (RBC) was increased in those with four α genes [111]. In this same Kenyan study the presence of sickle cell trait was found to ameliorate the effect of α thalassaemia trait on the RBC, Hb, MCV and MCH [111]. α thalassaemia trait is somewhat

Table 4.4 The red cell indices and percentage of haemoglobin S reported in sickle cell trait with and without deletion of α globin genes [13, 38, 110–115].

	$\alpha\alpha/\alpha\alpha$	$-\alpha/\alpha\alpha$	$-\alpha/-\alpha$	Method	Reference
Haemoglobin S percentage	>38	31–38	< 31	DEAE-cellulose chromatography	[38]
	35–45*	30–35*	25–30*	Not stated*	[110]
	34–38	28–34	20–28		[13]
	31–43	29–35	22–29	HPLC	[113]
MCV (fl)	80–90	75–85	70–75	Not stated*	[110]
Range or mean (SD)	84.9 (8.26)	79.1 (5.8)	67.8 (5.81)	HPLC	[113]

* These figures are averages derived from published series and in which the α chain deletion was $-\alpha^{32}$; the quantification is likely to have been by electrophoresis; the deletion $-\alpha^{42}$ leads to a greater reduction in haemoglobin S percentage [114].

more prevalent in Africans and African Americans with sickle cell trait than in those with normal β globin genes [116] so that it is not rare for subjects with sickle cell trait to have borderline anaemia or reduction of the MCV and MCH.

Children with sickle cell trait and *falciparum* malaria have less reduction of the Hb, haematocrit, reticulocyte count and platelet count than children with *falciparum* malaria but without a haemoglobinopathy [117].

Blood film

The blood film may be completely normal or may show microcytosis or target cells (Fig. 4.3). If a subject with sickle cell trait develops iron deficiency, target cells are often prominent. Although classic sickle cells are only very rarely seen, small numbers of plump cells that are pointed at both ends have been reported [118]; such cells were described in about 96% of individuals with sickle cell trait in comparison with 4% of normal subjects. In very unusual circumstances, sickle cells are seen; this has been reported as an *in vitro* artefact in a child with acute lymphoblastic leukaemia with a high white cell count (WBC), being attributable to consumption of oxygen by the leukaemic cells [119].

During *P. falciparum* malaria, subjects with sickle cell trait have a blood film showing a lower percentage of parasitised cells than is seen in subjects without a haemoglobinopathy [117, 120].

Other investigations

It might be expected that heterozygotes for haemoglobin S ($\beta\beta^S$) would have equal amounts of haemoglobin S and haemoglobin A. In fact, haemoglobin A is somewhat more than 50% and haemoglobin S is somewhat less, usually around 40%; this is because normal β chain has a greater affinity than β^S for α chains. Haemoglobin electrophoresis at alkaline or acid pH shows a variant haemoglobin with characteristic mobility (Fig. 4.4). Haemoglobins D and G have the same electrophoretic mobility

at alkaline pH but can be distinguished by electrophoresis at acid pH, which usually shows mobility that is the same as, or very similar to, that of haemoglobin A. Haemoglobin S can also be distinguished from haemoglobin A, D and G by isoelectric focusing and by HPLC (Fig. 4.5), see also Fig. 2.19) and capillary electrophoresis (Fig. 4.6). A sickle solubility test (see Fig. 2.25) should always be performed when the presence of a significant proportion of haemoglobin S is suspected. It will be positive except in the early neonatal period when the percentage will be below the detection limit. It follows that a negative sickle solubility test in a neonate with a variant haemoglobin consistent with haemoglobin S by no means excludes a diagnosis of sickle cell trait. Reagents suitable for a sickle solubility test are commercially available in kit form.

Haemoglobin S can also be distinguished from haemoglobin A and from haemoglobin C by immunological techniques based on monoclonal antibodies to sequences including the amino acids that are substituted in haemoglobin S and haemoglobin C. It is thus possible to distinguish sickle cell trait from sickle cell anaemia and from sickle cell/haemoglobin C disease. However, sickle cell/ β^+ thalassaemia may not be distinguished from sickle cell trait.

The proportion of haemoglobin S can be quantified by scanning densitometry of an electrophoretic strip, by elution from an electrophoretic strip, by capillary electrophoresis or by HPLC. It should be noted that when the haemoglobin S percentage is estimated by HPLC rather than electrophoresis, there is underestimation as glycated haemoglobin S and other post-translationally modified S is excluded from the estimate. The percentage shows a trimodal distribution, depending on whether there is coexisting α thalassaemia trait (see Table 4.4). If there is coexisting haemoglobin H disease the percentage of haemoglobin S is even lower, around 10–25% [114], which sometimes leads to a false negative sickle solubility test. Conversely, if there are five α genes ($\alpha\alpha\alpha/\alpha\alpha$) the haemoglobin S percentage is somewhat higher than in those with a normal complement of α genes [121], about 45% rather

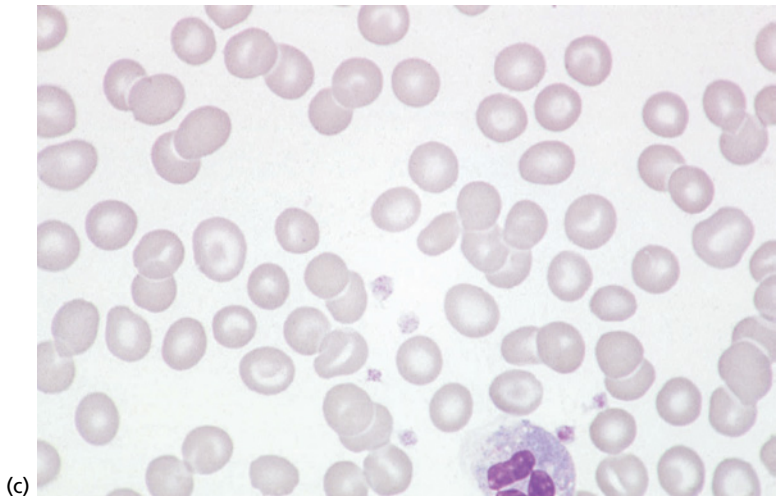
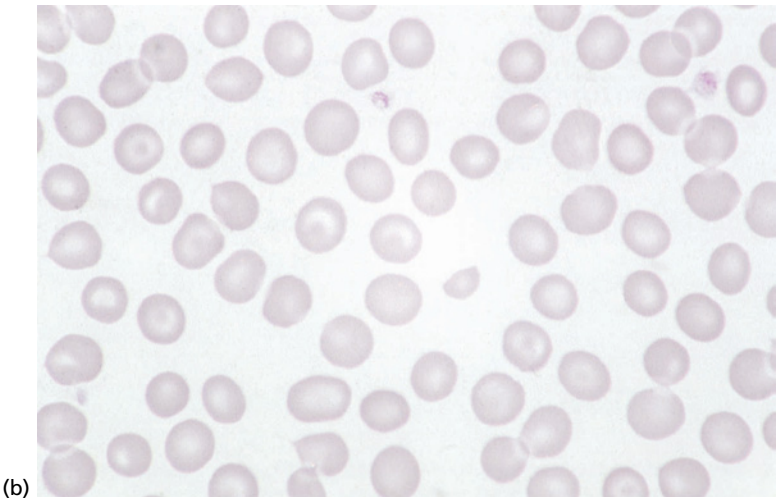
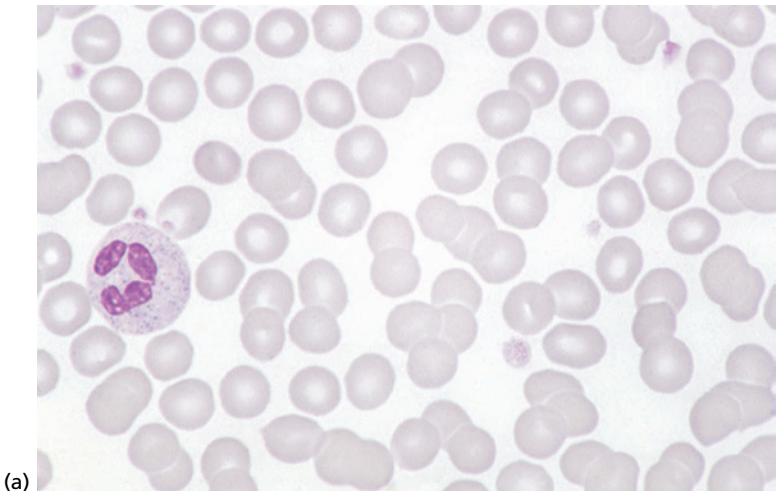


Fig. 4.3 Blood films from three patients with sickle cell trait showing the range of features observed: (a) normal film; (b) minimal anisocytosis and poikilocytosis with occasional target cells; (c) hypochromia with occasional target cells and other poikilocytes. May–Grünwald–Giemsa (MGG) $\times 100$ objective.

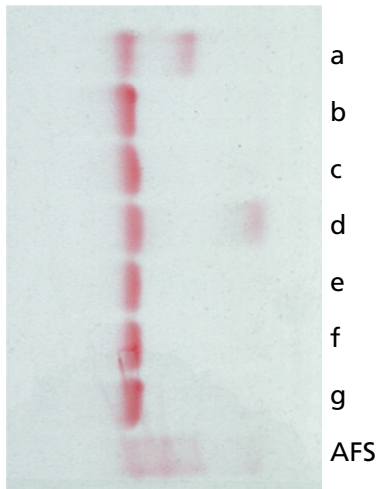


Fig. 4.4 Haemoglobin electrophoresis on cellulose acetate at alkaline pH showing haemoglobins A and S in a patient with sickle cell trait (lane a); AFS indicates a control sample containing haemoglobins A, F and S.

than about 40%. A rare cause of a low haemoglobin S percentage (11%) in sickle cell trait is coinheritance of a β thalassaemia determinant in *cis* (i.e. on the same chromosome); haemoglobin S may then be as low as 10% [122, 123]. A similarly low haemoglobin S percentage in a heterozygote (12%) has been reported as the result of somatic deletion of the β^s allele of the *HBB* gene in about 50% of cells [124]. A further rare cause of a low S percentage (13.5%), observed in a patient with deletion of a single α gene, was duplication of the entire β globin cluster in *trans* [125]. The percentage of haemoglobin S correlates with the MCV and MCH since all these variables are influenced by coexisting α thalassaemia trait (see page 218). The proportion of haemoglobin S is also reduced if there is coexisting iron deficiency [126], has been observed to fall markedly in megaloblastic anaemia [127] and may be low in lead poisoning [128].

The percentage of haemoglobin A_2 may be slightly elevated in sickle cell trait [113, 129]. In addition, the A_2 fraction on HPLC is artefactually increased by the presence of carbamylated [130] or other post-translationally modified haemoglobin S. Quantification of haemoglobin

A_2 is not a particularly useful investigation to perform in an individual with sickle cell trait so this is not of practical importance. In one study, haemoglobin F was slightly increased with a mean of 1.4%; F cells were a mean of 14.1% in comparison with a mean of 2.8% in haematologically normal African Americans [131].

In the neonatal period haemoglobin F will be present in large amounts. There will be more haemoglobin A than haemoglobin S. However, if haemoglobins S and A are present in small amounts then precise quantification may be difficult. Unless there is clearly more haemoglobin A than S then sickle cell/ β^+ thalassaemia is a possible alternative diagnosis, and deoxyribonucleic acid (DNA) analysis should usually be performed to clarify the diagnosis. Alternatively, the diagnosis may be made by testing the parents and other family members, or the test can be repeated when the infant is a few months old.

Electrophoretic features suggestive of sickle cell trait despite clinical features of sickle cell disease should lead to investigation for an electrophoretically silent variant haemoglobin that may be interacting with haemoglobin S [53].

Diagnosis

Diagnosis rests on demonstration of the presence of haemoglobin S and haemoglobin A with the percentage of haemoglobin S being less than the percentage of haemoglobin A. The haemoglobin S identification must be supported by two independent tests.

Interactions of haemoglobin S heterozygosity with thalassaemias and haemoglobinopathies

The interaction of sickle cell trait and α thalassaemia trait has been discussed earlier. The coexistence of sickle cell trait and the genotype of haemoglobin H disease leads to a modification of the phenotype of the haemoglobin H disease. There is a hypochromic microcytic anaemia with splenomegaly and erythroid hyperplasia but with a normal

reticulocyte count [132]. The blood film shows hypochromia, microcytosis and marked poikilocytosis including target cells (Fig. 4.7). Haemoglobin S is lower than is usual in sickle cell trait with coexisting α thalassaemia trait (Fig. 4.8) [54, 132]. Haemoglobin Bart's may be present in infancy but haemoglobin H is not detected and only occasional inclusion-containing cells are found on a haemoglobin H preparation. Inclusions have been reported in bone marrow erythroblasts. It could be speculated that these represent β^S precipitates, β^A having combined preferentially with the reduced numbers of α chains.

The coinheritance of β^S and various β chain variants, β and $\delta\beta$ thalassaemias leading to a clinical abnormality is discussed later. Other compound heterozygous states may be asymptomatic. The interaction of sickle cell trait and α chain variants is generally clinically silent but leads to extra bands on haemoglobin electrophoresis and extra peaks on HPLC. For example, coinheritance of sickle cell trait and $\alpha^{G\text{-Philadelphia}}$ is associated, on electrophoresis at alkaline pH, with the presence of three bands with the mobility of A, S (representing S and G-Philadelphia) and C (representing an S-G hybrid) (Fig. 4.9). On agarose gel at acid pH

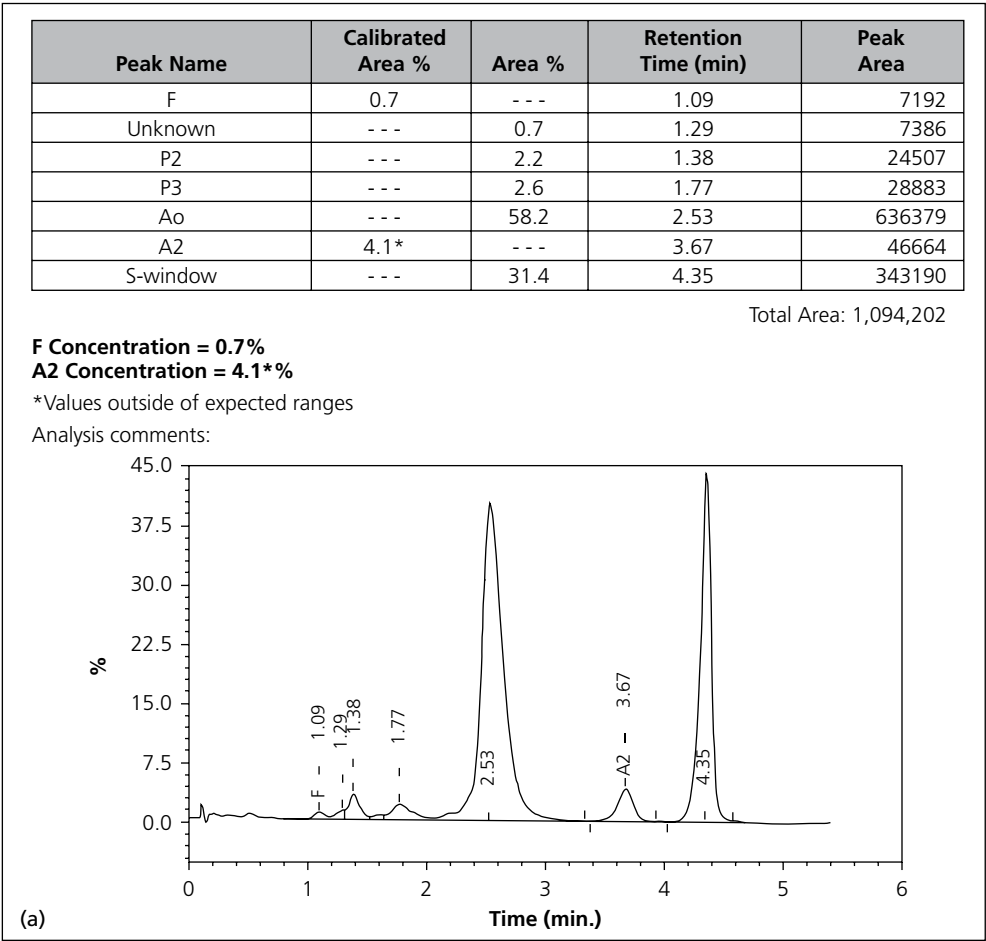


Fig. 4.5 High performance liquid chromatography (HPLC) chromatogram, Bio-Rad Variant II: (a) sickle cell trait in an adult showing, from left to right, haemoglobin F (shaded), two peaks of post-translationally modified haemoglobin A, haemoglobin A₀, haemoglobin A₂ (apparently increased) and haemoglobin S; (Continued on p. 223.)

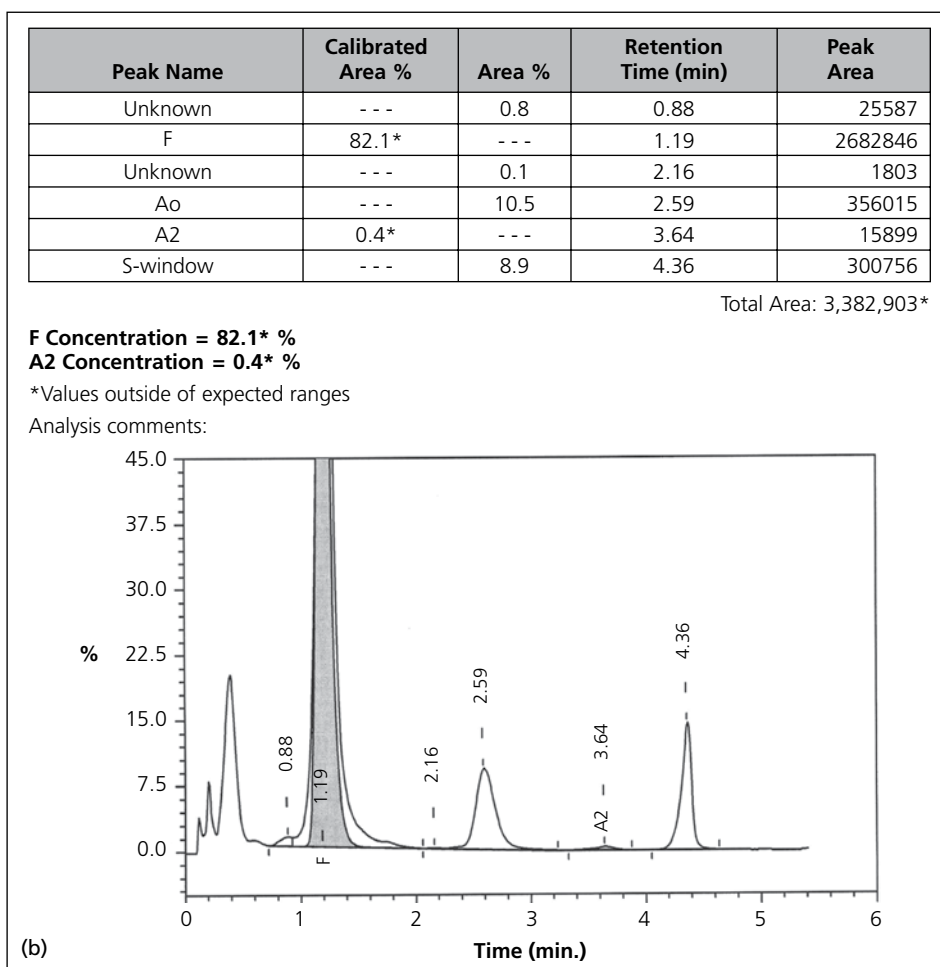


Fig. 4.5 *Continued.* (b) sickle cell trait in a neonate, showing haemoglobin F₀ 82.1%, haemoglobin A₀ 10.5%, haemoglobin A₂ 0.4% (the low level being expected in a neonate) and haemoglobin S 8.9%. The post-translationally modified F (complex peaks at left) has not been integrated.

there are two bands, a band with the mobility of haemoglobin A (representing A plus G-Philadelphia) and a band with the mobility of haemoglobin S (representing S and S-G hybrid). On HPLC there are four fractions distinguished by their retention times (Fig. 4.10).

Very rarely, a β thalassaemia mutation occurs in *cis* to a β^s mutation, resulting in an asymptomatic condition that differs considerably from sickle cell trait. Two individuals with this combination had haemoglobin S of 10–11%, haemoglobin A₂ of 6–7%, haemoglobin F of around 3% and a mild microcytic anaemia with a reticulocyte count of around 3% [123, 133].

Interactions of haemoglobin S heterozygosity with other haematological conditions

The coexistence of sickle cell trait and hereditary spherocytosis has been reported in at least 19 instances. Four of these individuals suffered either splenic sequestration or splenic infarction [134]. It is likely that in the increased haemoglobin concentration within red cells as a result of the hereditary spherocytosis favours sickling within the spleen. Such patients can also have functional hyposplenism despite an enlarged spleen (Fig. 4.11) [71]. Sickling can also

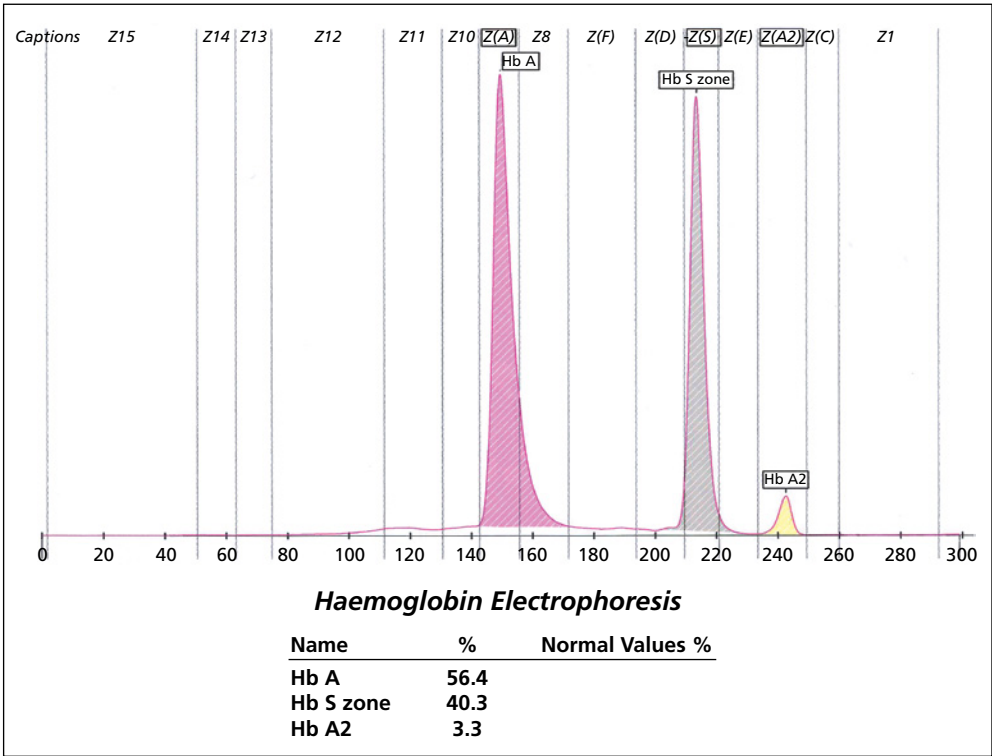


Fig. 4.6 Capillary electrophoresis, electropherogram (Sebia Capillarys 3) from a patient with sickle cell trait, showing haemoglobins A, S and A₂.

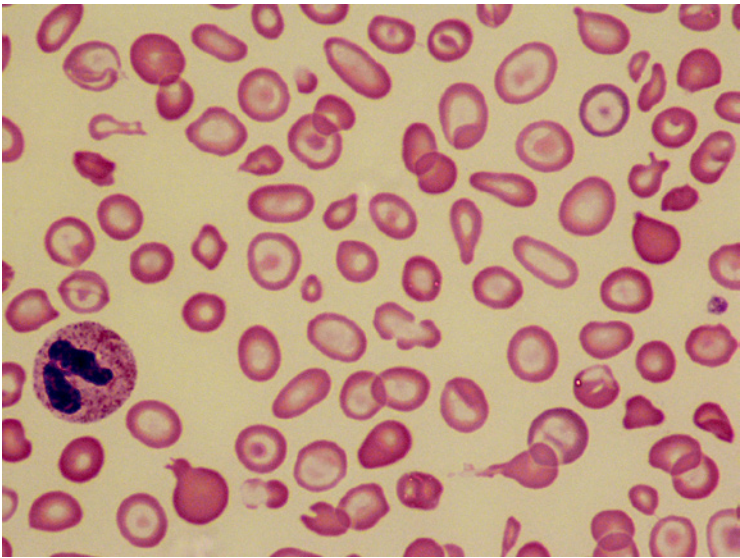


Fig. 4.7 Blood film of a patient with sickle cell trait and the genotype of haemoglobin H disease (homozygosity for Saudi nondeletional α thalassaemia (AATAAA \rightarrow AATAAG at the polyadenylation site, $\alpha^{\text{T Saudi}}\alpha / \alpha^{\text{T Saudi}}\alpha /$). Full blood count (FBC) showed: red blood cell count (RBC) $5.83 \times 10^{12}/\text{l}$, haemoglobin concentration (Hb) 97 g/l, haematocrit (Hct) 0.31, mean cell volume (MCV) 54 fl, mean cell haemoglobin (MCH) 16.6 pg, mean cell haemoglobin concentration (MCHC) 313 g/l. MGG $\times 100$.

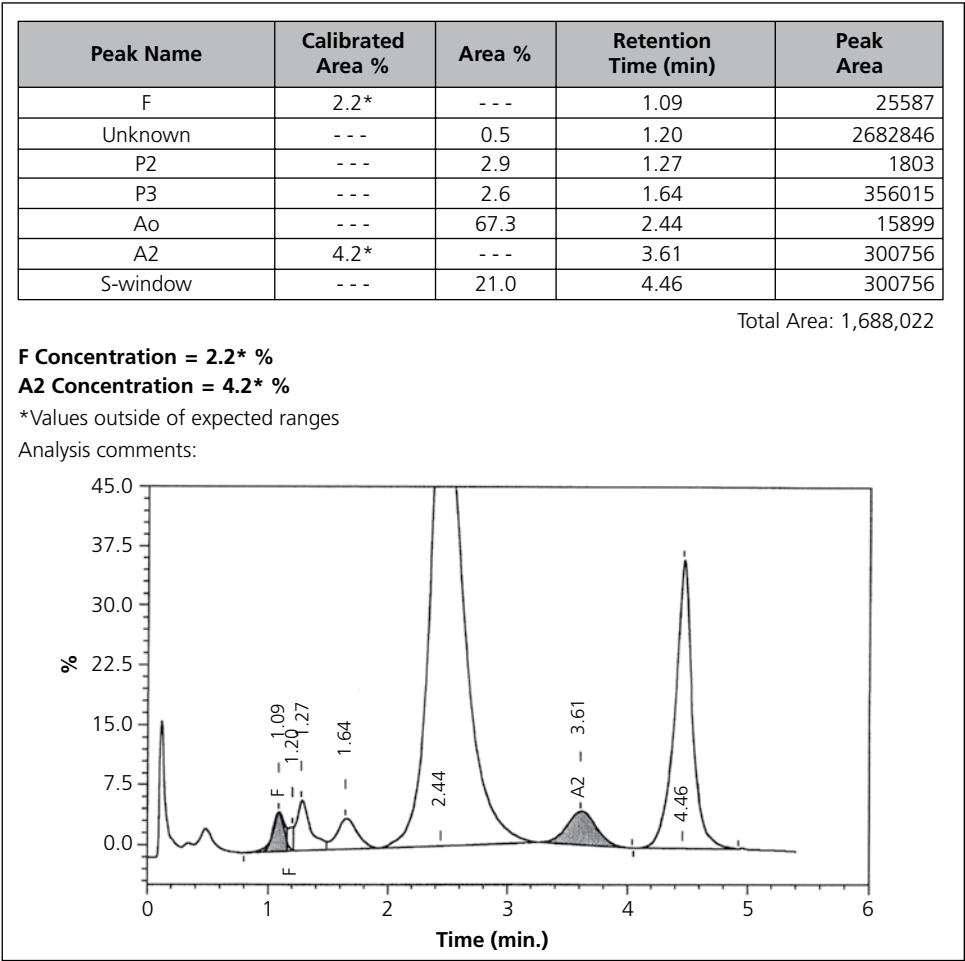
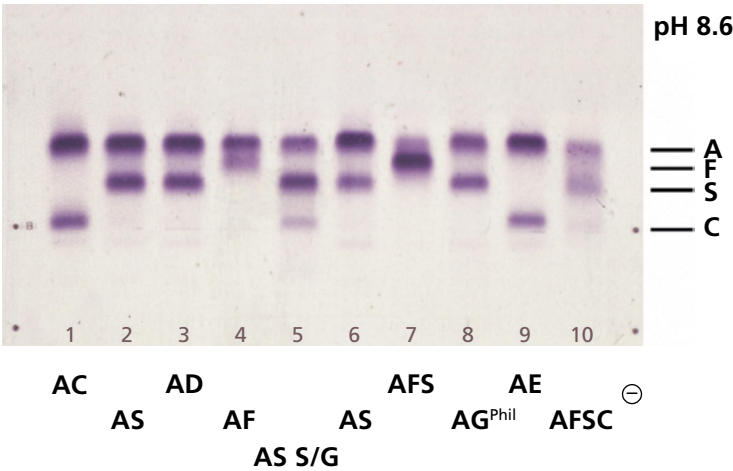


Fig. 4.8 HPLC chromatogram, Bio-Rad Variant II, in sickle cell trait plus haemoglobin H disease showing the typical double peak of haemoglobin H and a low haemoglobin S percentage (21%). (Same patient as Fig. 4.7.)

⊕

Fig. 4.9 Haemoglobin electrophoresis on agarose gel at pH8.6 showing three bands with the mobilities of A, S and C, in a patient with sickle cell trait and heterozygosity for haemoglobin G-Philadelphia (fifth lane from left); AFSC indicates a control sample containing haemoglobins A, F, S and C.



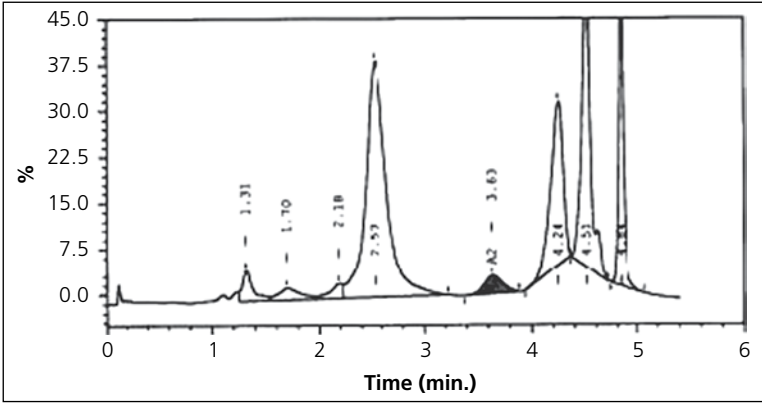


Fig. 4.10 HPLC chromatogram in a patient with heterozygosity for both haemoglobin S and haemoglobin G-Philadelphia showing, from left to right, post-translationally modified haemoglobin A (two peaks) and haemoglobins A_V, A₂, S, G-Philadelphia and hybrid (α^Cβ^S).

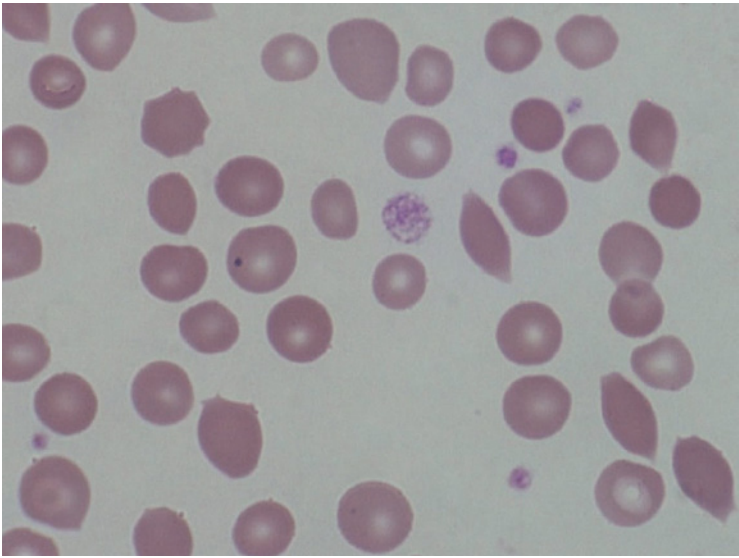


Fig. 4.11 PB film of a patient with coexisting sickle cell trait and hereditary spherocytosis showing spherocytes and several boat-shaped cells together with a large platelet and a Howell-Jolly body, indicating that there was hyposplenism despite the patient having splenomegaly. MGG $\times 100$.

occur when severe pyruvate kinase deficiency coexists with sickle cell trait; this is likely to be the result of a high intracellular concentration of 2,3-diphosphoglycerate (2,3-DPG) favouring deoxygenation [71].

Sickle cell anaemia

Sickle cell anaemia is the disease resulting from homozygosity for haemoglobin S. Individuals with sickle cell anaemia have haemoglobin S as the major haemoglobin component with a small proportion of haemoglobin A₂ and a variable proportion of haemoglobin F. Since there is no synthesis of normal β chain there is a total

absence of haemoglobin A. Red cells sickle due to polymerisation of haemoglobin S under conditions of low oxygen tension, and initially this process is reversible if the cells are reoxygenated. The process is typically repeated many times as the red cells circulate through the lungs and around the body, but eventually progressive membrane damage leads to the red cell becoming irreversibly sickled. The irreversibly sickled cell has an increased calcium content, which triggers Ca-dependent potassium transport and loss of potassium and water. K/Cl co-transport is also increased. The dehydrated cell becomes even more rigid. The red cell membrane is damaged by oxidation and

the effects of repeated polymerisation with clustering of band 3 protein and externalisation of phosphatidyl serine. There is reduced red cell deformability and increased fragility, leading to microvesiculation and intravascular haemolysis. Intravascular haemolysis releases free haemoglobin and haem into the circulation, which causes a functional loss of nitric oxide (NO); this is further aggravated by release of red cell arginase, which reduces availability of arginine, the substrate for NO synthesis, with a resultant loss of NO vasodilator function. The free plasma haem and haemoglobin also upregulate inflammasome activity, triggering an inflammatory cascade with high levels of interleukin-1b, interleukin-18 and other cytokines. Immunoglobulin G binds to the damaged red cell membrane and this, plus red cell rigidity, leads to phagocytosis by macrophages (extravascular haemolysis). Free haemoglobin S in the circulation may cause direct activation of monocytes leading to further increase in proinflammatory cytokines [135]. Sickle cells show increased interaction with endothelium, particularly when adhesion molecules are upregulated, and also increased binding to neutrophils and platelets. There is a prothrombotic state mediated by the altered red cell membrane, damaged endothelium, activated neutrophils, monocytes and platelets, and NO depletion. There is an increased incidence not only of arterial thrombosis but also of venous thromboembolism.

Red cell survival is around 20 days in most patients but can be considerably longer when haemoglobin F is significantly increased; hydroxycarbamide (hydroxyurea) may significantly improve red cell survival and one study found that in six patients taking hydroxycarbamide, with haemoglobin F levels of more than 10%, red cell survival was 16–54 days (mean 34 days) [136].

Sickle cell anaemia occurs worldwide but the majority of affected births occur in Nigeria, the Democratic Republic of the Congo and India [137]. Median survival in the UK has been estimated at 67 years [138] and in the USA at 48 years or 66 years, depending on the statistical methods employed [139]. Large cohort studies

from birth will be needed to establish accurate figures.

A Ugandan study found that sickle cell anaemia was significantly less prevalent in patients hospitalised with *falciparum* malaria than in the general population, indicating a protective effect [140]. Nevertheless, children with sickle cell anaemia are highly susceptible to the lethal effects of such infection. Splenomegaly is an unexplained risk factor for malaria [141]. The risk of malaria is reduced by hydroxycarbamide therapy, with the reduced risk correlating with a neutrophil count below $3 \times 10^9/l$ [141]. However, a higher haemoglobin F level favours *P. falciparum* proliferation [142].

It is unclear if there is an increased risk of malignancy associated with sickle cell disease. A study in California found an increased incidence of leukaemia in patients with sickle cell disease (not all patients necessarily having sickle cell anaemia) with a reduced incidence of breast cancer and male genital cancer [143]. A UK (English) study based on a coded national dataset found an increased incidence of some malignancies, although these findings have not been validated against accurate patient records [144]. A study of patients with sickle cell anaemia and sickle cell/ β^0 thalassaemia found an association with an increased prevalence and earlier onset of clonal haemopoiesis [145], although this was not confirmed in a second study [146].

The incidence of venous thromboembolism is increased in sickle cell disease (sickle cell anaemia plus sickle cell/ β thalassaemia), more so in patients who have been splenectomised [147].

A very rare cause of late onset of some features of sickle cell anaemia is mosaic segmental uniparental disomy for chromosome 11p13, causing a mixture of SS and AS cells [148].

Clinical features

The clinicopathological features of sickle cell anaemia result directly or indirectly from haemolysis and vascular obstruction by sickled red cells with consequent tissue infarction. In addition to the shape change, erythrocytes show increased adhesion to endothelium, which contributes to vascular occlusion. Neonates are

asymptomatic since a major part of the total haemoglobin is haemoglobin F. As synthesis of haemoglobin F decreases and synthesis of haemoglobin S increases, symptoms start to appear, although rarely before six months of age. In infants, bony infarction leads to avascular necrosis of small bones of the hands and feet which presents clinically as painful swelling of fingers and toes (dactylitis or 'hand-foot syndrome'). This can lead to failure of growth of a phalanx and later shortening of a digit, although this is very rare (Fig. 4.12). In children there can be splenomegaly and occasionally there is hypersplenism. Young children can also suffer from splenic sequestration in which pooling of red cells in a rapidly enlarging spleen leads to acute anaemia. Splenic sequestration is not uncommonly followed by hypersplenism [149]. A traumatic splenic rupture has been reported [150–152]. In a study of 100 children aged 0–16 years, ultrasonography showed no detectable spleen in 5.6% of children aged 6–10 years and in 19.4% of children aged

11–16 years [153]. Overall, 28% had a spleen that was large for the age of the child. [153]. Recurrent splenic infarction can lead to calcification of a normal sized spleen (Fig. 4.13) or a small atrophic spleen (Fig. 4.14). Hepatic sequestration also occurs but is less common. Cerebral infarction is a particular feature of children with sickle cell anaemia, as a result of prior endothelial damage, typically causing narrowing of the middle cerebral artery. Silent cerebral infarcts also occur, with the frequency being higher with a lower Hb and also correlating with previous parvovirus B19 infection [154]. Cerebral fat embolism can present with non-focal neurological manifestations, such as an altered level of consciousness, and is sometimes part of a more general fat embolisation syndrome, with the development of acute chest syndrome and a high risk of death [155]. There is also an increased incidence of intracranial aneurysms. Hearing loss is common, due either to otitis media with effusion or to sensorineural loss [156]. Tinnitus and sudden deafness can occur.

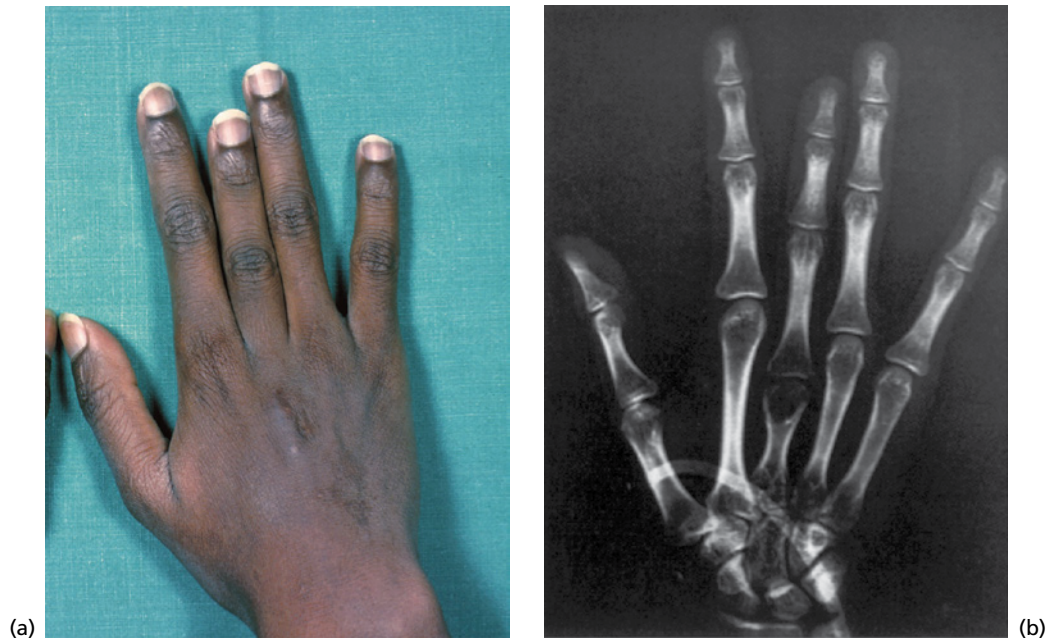


Fig. 4.12 Long term result of 'dactylitis' in sickle cell anaemia: (a) the hand of an 18-year-old Nigerian man; (b) radiograph of the hand. (Reproduced from Hoffbrand AV and Pettit JE. *Essential Haematology*, 3rd edn. Blackwell Scientific Publications, Oxford, 1993, by kind permission of Professor Victor Hoffbrand and the publisher.)



Fig. 4.13 Computed tomography of the abdomen of a patient with sickle cell anaemia showing calcification of a normal sized spleen.



Fig. 4.14 Computed tomography of the abdomen of a patient with sickle cell anaemia showing calcification of a small (atrophic) spleen.

In older children and adults there continues to be infarction of bones such as ribs, vertebrae and long bones; osteonecrosis can be detected radiologically (Fig. 4.15a). Skull infarction leading to extradural haematoma formation is rare but often fatal [157]. In addition, there is

progressive damage of internal organs including the lungs (Fig. 4.15b), abdominal organs and brain. The incidence of acute painful episodes correlates with a higher haematocrit and the incidence of acute chest syndrome with cigarette smoking [158]. Painful episodes can be

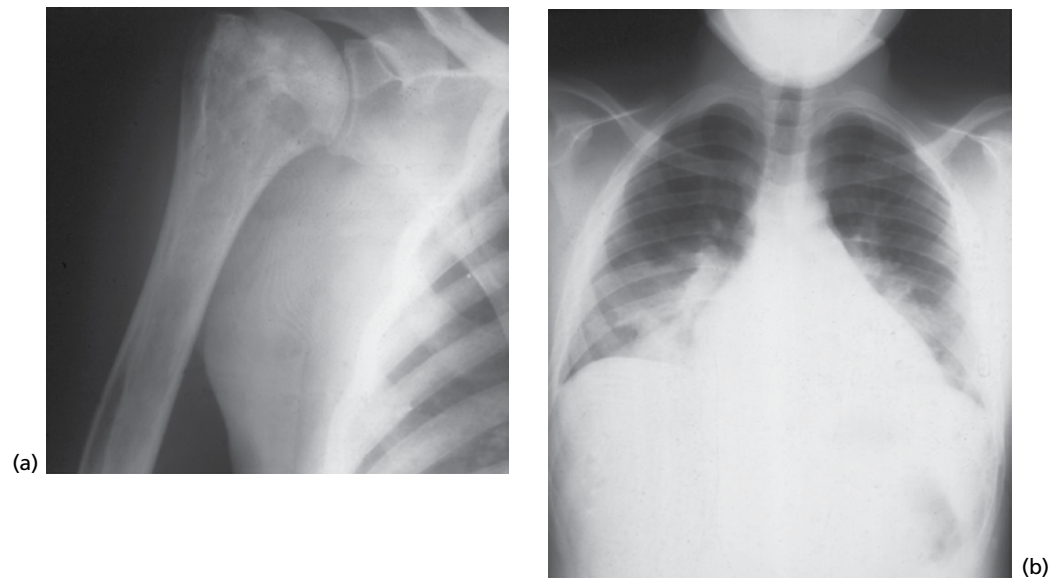


Fig. 4.15 Radiography in sickle cell anaemia: (a) radiograph of the head of the humerus showing areas of reduced radiodensity, consequent on previous infarction; (b) chest radiograph showing opacities in the lower half of both lung fields representing pulmonary infarction as a result of sickle cell formation and vascular occlusion. (With thanks to Professor Irene Roberts, Oxford and London.)

Table 4.5 Features of sickle cell anaemia linked to haemolysis and anaemia.

Linked to haemolysis/ anaemia	Linked to less anaemia
Leg ulcers	Painful crises
Priapism	Acute chest crises
Pulmonary hypertension	Retinopathy
Albuminuria and chronic kidney disease	Osteonecrosis
Gallstones	

precipitated not only by infection and hypoxaemia but also by exposure to corticosteroids [159]. Retinopathy can occur, a haemoglobin F of at least 15% being protective [160]. Some clinical features of sickle cell anaemia are associated with the rate of haemolysis while others are linked more to blood viscosity (Table 4.5). Inactivation of NO by reactive oxygen species and by free haemoglobin in the plasma is likely to be a major cause of pulmonary hypertension [161], the prevalence of which correlates with the severity of

haemolysis [162]. Pulmonary infarction, resulting from acute chest syndrome and thromboembolism, can be associated with pulmonary sequestration of red cells and platelets and, if recurrent, can contribute to lung fibrosis, pulmonary hypertension and right heart failure with tricuspid incompetence. Pulmonary fibrosis aggravates hypoxia. Diffuse myocardial fibrosis can occur, leading to diastolic dysfunction [163]. Other cardiac complications include atrial and ventricular dilation, atrial arrhythmias, mitral incompetence and right to left shunting, either in the lung or through a patent foramen ovale, with associated hypoxia [164]. Bone marrow infarction can be extensive and may be complicated by embolism of necrotic bone marrow to the lungs. Osteomyelitis is more common in sickle cell disease, possibly related to bone marrow infarction, with *Salmonella* species accounting for nearly 50% cases and *Staphylococcus aureus* occurring in 20%. Recurrent infarction of the spleen leads to hyposplenism, which in turn causes increased severity of various infections including malaria and pneumococcal

septicaemia. Splenic phagocytic function is lost first and then splenic filtering function [165]. In one study, loss of splenic function began before 12 months of age in 86% of 193 babies [166]. Autosplenectomy can occur with no splenic tissue being detected on imaging [167]. Acquired von Willebrand disease related to extreme thrombocytosis has been reported [168]. In developed countries, splenomegaly is seen mainly in young children (in the steady state due to congestion and extramedullary haemopoiesis) but in sub-Saharan Africa splenomegaly is common in the first 10 years of life and is observed even in 10% of adolescents [169] and also in adult life [167]; a relationship to malaria and other recurrent infections is uncertain [167, 169]. Persistent splenomegaly is also often observed with the Arab-Indian haplotype [170]. Infarction of the skin can result in ulceration of the legs (Fig. 4.16); this



Fig. 4.16 Clinical photograph showing ulceration of the lower leg in a patient with sickle cell anaemia.

is more common in low- and middle-income countries so other factors are clearly also operating. The increased breakdown of red cells means that patients are intermittently jaundiced (Fig. 4.17). There is a high incidence of pigment gallstones (Fig. 4.18) occurring at a young age, resultant on chronic haemolysis, which in turn results in an increased incidence of pancreatitis. The gallstones may be radiolucent or radio-opaque so are best detected by ultrasonography. The coinheritance of Gilbert syndrome aggravates hyperbilirubinaemia. Acute hepatic complications can occur, characterised by pain and tenderness in the right upper quadrant, fever and vomiting [171]; this involves a range of different underlying pathological processes, sometimes called sickle hepatopathy, and can be complicated by coagulopathy, liver failure and death [171]. Increased erythropoiesis occurs as a response to haemolytic anaemia, and to a lesser extent ineffective erythropoiesis, leading to overexpansion of the bone marrow cavity and a tendency to reduced bone mineral density. In some patients erythroid hyperplasia causes frontal bossing of the skull and malpositioned teeth. On skull radiology there can be thickening of the cranial bones (Fig. 4.19) and a hair-on-end appearance.

Patients with sickle cell anaemia can suffer rapid worsening of anaemia, with Hb sometimes falling to 40 g/l or lower, during infection by parvovirus B19. The mechanism is pure red cell aplasia, which is transient but, because of



Fig. 4.17 Face of a child with sickle cell anaemia showing pallor and jaundice. (With thanks to Professor Irene Roberts.)



Fig. 4.18 Cholecystogram, showing gallstones (negative images) caused by increased bilirubin production resulting from haemolysis in a child with sickle cell anaemia. (With thanks to Professor Irene Roberts.)



Fig. 4.19 Skull radiograph in sickle cell anaemia showing expansion of the bony cavity resulting from hyperplastic erythropoiesis.

the shortened red cell life span, rapidly leads to anaemia. In some countries, patients with sickle cell anaemia show an increased incidence of megaloblastic anaemia, which has been attributed to inadequate intake of folic acid in the face of an increased need for this vitamin. Transfusion-transmitted babesiosis can cause severe haemolysis [172].

Patients with sickle cell anaemia have an increased incidence of pregnancy complications including more frequent pre-eclampsia/eclampsia, need for induction of labour, caesarean section, preterm birth and admission of the neonate to a neonatal unit [173]. Thromboembolism is also significantly higher than in control patients [174].

Patients with sickle cell anaemia have an increased incidence of renal disease, which is present from an early age and progresses to cause renal failure in 30% of older adults. Features include hyperfiltration with an increased glomerular filtration rate early in the disease course (from early childhood up until the age of about 30 years), glomerular hypertrophy, glomerulopathy (focal segmental or membranoproliferative glomerulonephritis or microangiopathic glomerulopathy) and papillary necrosis or other renal infarction [175]. Functional consequences include nocturnal enuresis, haematuria, microalbuminuria (loss of 30–300 mg of albumin per day), albuminuria (loss of more than 300 mg per day, reported in 26–68% of patients [175]), nephrotic syndrome, loss of concentrating ability and chronic renal failure. Hyperfiltration may lead to microalbuminuria and it is possible that it contributes to glomerular damage [176, 177]. A decline in estimated glomerular filtration rate with increasing age may represent declining kidney function rather than an improvement in hyperfiltration. An Hb of less than 80 g/l correlates with earlier development of microalbuminuria [178]. Leucocytosis and the degree of haemolysis also correlate with earlier onset of albuminuria [177]. A study of patients with sickle cell disease, three-quarters of whom had sickle cell anaemia, found that pre-eclampsia was associated with a decline in renal function persisting after the end of pregnancy [179]. Ovarian reserve is reduced in

young women with sickle cell anaemia, this correlating with hydroxycarbamide use [180].

Patients with sickle cell anaemia living at a high altitude have a somewhat higher mean Hb and higher 2,3-DPG and are considerably more symptomatic than those living at low altitudes [181].

Patients with sickle cell anaemia with coexisting autoimmune disease have a higher prevalence of leg ulcers and more vaso-occlusive events [182] and are more likely to suffer severe and progressive complications, possibly related to chronic treatment with corticosteroids.

Patients with sickle cell anaemia with a high haemoglobin F (e.g. those with the Arab-Indian haplotype) generally have a milder clinical course but severe disease can occur. There is significant retention of splenic function but the associated persisting splenomegaly means that they can develop hypersplenism and remain susceptible to splenic sequestration, splenic infarction and splenic abscess formation into adult life [183]; splenectomy may be necessary for hypersplenism. Because there is less haemolysis, there may be a lower incidence of pulmonary hypertension, priapism, leg ulcers, stroke and nephropathy [131, 184, 185] whereas the incidence of vaso-occlusive events may be similar or higher [32, 184]. There is a reduction in acute chest syndrome and painful crises and an improved life expectancy [185]. In one study, avascular necrosis of the femoral head occurred in around 27% of patients in comparison with 8–12% in patients with a lower haemoglobin F level [32]. There is some evidence that a higher haemoglobin F percentage is associated with a higher rate of death from *falciparum* malaria [186] but a markedly reduced incidence of malaria has been observed in relation to a higher haemoglobin F level associated with hydroxycarbamide therapy [185]. It is likely that the protective effect of a higher haemoglobin F percentage is related to the percentage of F cells with a protective level of haemoglobin F rather than the proportion of F cells or the overall percentage of haemoglobin F [185].

Patients who require regular or intermittent blood transfusions often develop red cell alloantibodies and are thus at risk of haemolytic

transfusion reactions, which can be severe and associated with intravascular haemolysis. Such reactions may be accompanied by acute vaso-occlusive pain, acute chest syndrome, pulmonary hypertension and multiorgan failure [187]. When a delayed transfusion reaction occurs, the Hb may fall rapidly to levels below the pretransfusion level. This is due mainly to destruction of transfused red cells while haemopoiesis is suppressed but in some patients there is also 'bystander' destruction of the patient's own red cells [188] leading to severe anaemia and haemoglobinuria, sometimes referred to as hyperhaemolysis. In addition, hyperhaemolysis can occur without any evidence of red cell incompatibility [189], resulting from macrophage activation and associated with high levels of ferritin and interleukin 6; its recognition is important since it can cause very severe anaemia. Treatment options include tocilizumab (targeted at the interleukin 6 receptor [190]), rituximab (targeted at CD20-expressing B cells) and eculizumab (targeted at complement C5).

The causes of anaemia in homozygotes for haemoglobin S are summarised in Table 4.6 [191]. Haemolysis can be aggravated by coinheritance of hereditary elliptocytosis [192].

Table 4.6 Causes of anaemia in sickle cell anaemia.

Causes of steady state anaemia

Haemolysis

Reduced oxygen affinity leading to reduced erythropoietic drive

Ineffective erythropoiesis

Causes of worsening of anaemia

Splenic, hepatic or pulmonary sequestration

Acute chest syndrome

Hypersplenism (usually only in infants and children)

Parvovirus B19 infection

Suppression of erythropoiesis in other infections

Malaria and, rarely, babesiosis

Megaloblastic anaemia resulting from folic acid deficiency or vitamin B₁₂ deficiency as a result of chronic use of N₂O for pain relief [191]

Bone marrow infarction

Haemolytic transfusion reactions

Development of coexistent diseases

Renal failure

Death in sickle cell anaemia is most often attributable to infection, cerebrovascular accidents, renal failure or cardiopulmonary disease, the latter being the result of extensive sickling within pulmonary blood vessels. In the absence of parental education and vigilance, death of infants can result from splenic sequestration. In African countries it is likely that many deaths in infants and children with sickle cell anaemia are attributable to malaria or other infections, and some to severe anaemia. Patients with sickle cell anaemia who survive childhood and adolescence may die from end organ failure due to recurrent tissue infarction (e.g. from renal or hepatic failure).

Patients with sickle cell anaemia can suffer complications related to treatment as well as those due the disease itself. These include transfusion-transmitted infections and iron overload.

In many countries the survival of individuals with sickle cell anaemia has improved greatly in recent decades. In one US study, published in 1994, median life expectancy was 42 years for men and 48 years for women [193] and in a Jamaican study from a similar period it was 58 years for men and 66 years for women [194]. By 2014, the estimated median survival in a US cohort was 58 years [195], and in 2016 a study suggested a median survival of 67 years in London, UK [138]. Despite the severity of the disease in many patients, there are a significant minority who are asymptomatic for prolonged periods. For example, in one French study of 299 patients, 9% were asymptomatic for three years or more [196]. In sub-Saharan Africa the situation is very different. It has been estimated that there is a 50–90% mortality by the age of five years, as a result of infection and severe anaemia [197].

In one study, sickle cell anaemia caused a significantly increased risk of hospitalisation and death from SARS-CoV-2 infection [106], although the increased risk seems to apply mostly to adults, with few studies finding a higher rate of complications in children with sickle cell disease.

Ameliorating factors and interaction with α thalassaemia trait

The clinical course of sickle cell anaemia is very variable. This is largely unexplained although

some factors have been identified that appear to ameliorate the condition and lead to later presentation, milder symptoms and a better life expectancy. Some of these are shown in Table 4.7 [6, 121, 165, 185, 193, 198–205]. As detailed earlier, higher haemoglobin F percentages protect against some complications of sickle cell anaemia, although there is less protection against other complications, such as cerebrovascular disease [131, 184]. Elevation of haemoglobin F to more than 20% is usually sufficient to render sickle cell anaemia largely asymptomatic.

Table 4.7 Factors ameliorating or aggravating features of sickle cell anaemia [6, 121, 165, 185, 193, 198–205].

Coinheritance of hereditary persistence of fetal haemoglobin, or other factors either linked or unlinked to the β globin locus, leading to a high percentage of haemoglobin F ameliorates; haemoglobin F level is highest in the Saudi Arabian/Indian and Senegal haplotypes (respectively averaging 17% in adults and 30% in children with the Arab-Indian haplotype and 10% with the Senegal haplotype [185]), lowest in the Central African Republic and Cameroon haplotypes (averaging 8% in Arabs and 5% in Africans [185]) and intermediate in the Benin haplotype (averaging 11% in Arabs and 6% in Africans [185])
Coinheritance of certain α chain variant haemoglobins, e.g. haemoglobin Memphis or haemoglobin Hopkins II
Coinheritance of α thalassaemia trait – ameliorates haemolysis [199]; is associated with a higher Hb [199, 200]; ameliorates soft tissue end organ damage [121], reduces leg ulcers [198, 203], reduces the frequency of stroke [196, 203], is associated with longer preservation of splenic function [165] but with more splenomegaly [198, 203], reduces the incidence of priapism [204] – however, does not ameliorate painful crises [200] and probably increases their frequency [196, 199]; decreased the frequency of acute chest crisis in one study [198]; increases the frequency of retinopathy [203] and osteonecrosis [6, 203]; does not improve survival [193]; reduces cholecystitis and need for cholecystectomy but not the incidence of gallstones [205]
Iron deficiency [201] (ameliorates haemolysis)

Coexisting α thalassaemia trait is common in sickle cell disease. For example, 30% of African Americans with homozygosity for haemoglobin S have a single α gene deletion and 5% have two α genes deleted [202]. The effect of coexisting α thalassaemia trait is complex with some features being ameliorated and other being worsened. The complexity may be the result of two conflicting effects: (i) reduced polymerisation leading to less membrane damage, fewer dehydrated and irreversibly sickled cells and improved red cell survival and (ii) higher Hb, leading to increased blood viscosity. Features that are ameliorated by coinheritorship of α thalassaemia include cerebrovascular disease, priapism, leg ulceration, albuminuria and gallstones. In a US study, deletion of two α genes was associated with an increased prevalence of avascular necrosis, retinopathy and splenomegaly and a decreased prevalence of leg ulcers and cerebrovascular accidents [203]. In a study of sickle cell anaemia associated with the Arab-Indian haplotype, a tribal Indian group with a very high incidence of α thalassaemia trait had significantly fewer painful crises, infections and episodes of hospitalisation than a non-tribal group with a much lower incidence of α thalassaemia trait [206]. In a study in Cameroon, coinheritorship of $-\alpha^{3.7}$ heterozygosity or homozygosity was found to be associated with later diagnosis of sickle cell anaemia and possibly with longer survival [207]. However, in a large study of sickle cell anaemia in a population with the β^S gene associated with a variety of haplotypes overall life expectancy was not altered by coexisting α thalassaemia [193], so it appears likely that the beneficial and adverse effects of coexisting thalassaemia trait balance out.

Laboratory features

Blood count

The blood count is normal at birth. During the first year, as haemoglobin F is replaced by haemoglobin S, there is a fall of Hb and a rise in the reticulocyte count. Mean values differ from controls by 1–2 months of age [208, 209].

Anaemia and reticulocytosis continue throughout childhood, adolescence and adult life. The Hb reported in adults is most often between 60 and 100 g/l but can range from 50 to 120 g/l or even higher. In a personally observed series of 29 mainly African Caribbean patients the Hb ranged from 76 to 138 g/l with a mean of 90 g/l. In males there is a significant post-pubertal rise in the Hb averaging between 10 and 20 g/l [13]. Patients with a higher percentage of haemoglobin F tend to have a higher Hb [13]. The Hb is of some prognostic significance [193]. In infants, anaemia and a higher reticulocyte count are predictive of a higher rate of death and stroke (increasing risk with absolute reticulocyte counts in quartiles of <105, 105–193, 194–307, $>307 \times 10^9/l$), but anaemia lost its significance in multivariate analysis [210]. In this study a higher WBC (which is usually only noted after the age of six months) was not predictive of outcome [210]. In another study a neutrophil count of $10 \times 10^9/l$ or higher was of adverse prognostic significance [211]. The monocyte count is on average higher than in control subjects [212]. During complications such as splenic sequestration, parvovirus infection or megaloblastic anaemia the Hb may fall to as low as 15–30 g/l. Other infections, including influenza and bacterial infections, are also associated with some worsening of the anaemia. In older patients with sickle cell anaemia, a slow fall in the Hb without any alteration in the red cell indices may be found to be the result of the onset of renal failure. A fall in both the Hb and the reticulocyte count, associated with a fall in erythropoietin concentration, can be an early sign of the onset of renal insufficiency [213]. Although the reticulocyte count is elevated in sickle cell anaemia, usually to 5–20%, it is not increased in proportion to the reduction in Hb. This is partly because haemoglobin S has a lower oxygen affinity than haemoglobin A (P_{50} of about 35.4 mmHg [214] in comparison with a P_{50} for haemoglobin A of about 26.8 mmHg) and the drive to erythropoiesis is therefore less than would be anticipated from the Hb. For the same reason, serum erythropoietin concentration is lower than would be expected for the degree of

anaemia [215]. The increased P_{50} of haemoglobin S is dependent on polymerisation of the haemoglobin, non-polymerised haemoglobin S having a normal oxygen affinity [216]. In addition, the concentration of 2,3-DPG is increased in sickle cell anaemia [184]. Ineffective erythropoiesis also contributes to the relatively low reticulocyte count for the degree of anaemia [217]. In patients with no associated α thalassaemia the red cell indices are normal [198, 218]. However, the MCV and MCH are not elevated in keeping with the reticulocyte count, suggesting a relative microcytosis. The MCV tends to be higher in patients with a higher haemoglobin F percentage [13] and is usually high in those taking hydroxycarbamide. The mean cell haemoglobin concentration (MCHC) may be slightly increased and the proportion of cells with a high haemoglobin concentration is increased (Fig. 4.20). The red cell distribution width (RDW) is generally markedly increased

and correlates with disease severity [219]. The total nucleated cell count may be increased as a consequence of significant numbers of circulating erythroblasts. The neutrophil count may be increased between as well as during acute illness. The baseline WBC has been found to correlate with the frequency of acute chest syndrome [220] and silent cerebral infarcts ($WBC \geq 11.8 \times 10^9/l$) [221] and to be associated with earlier death from sickle cell disease [193]. The baseline WBC and neutrophil count are also associated with deterioration of pulmonary function [222]. An increased WBC, partly hereditary and partly related to cigarette smoking, correlates with the incidence of priapism [158]. The monocyte count and the lymphocyte count are also increased [223], the latter possibly as a feature of hyposplenism. The platelet count is increased and there is an increased proportion of large platelets. Both these features are attributable to hyposplenism.

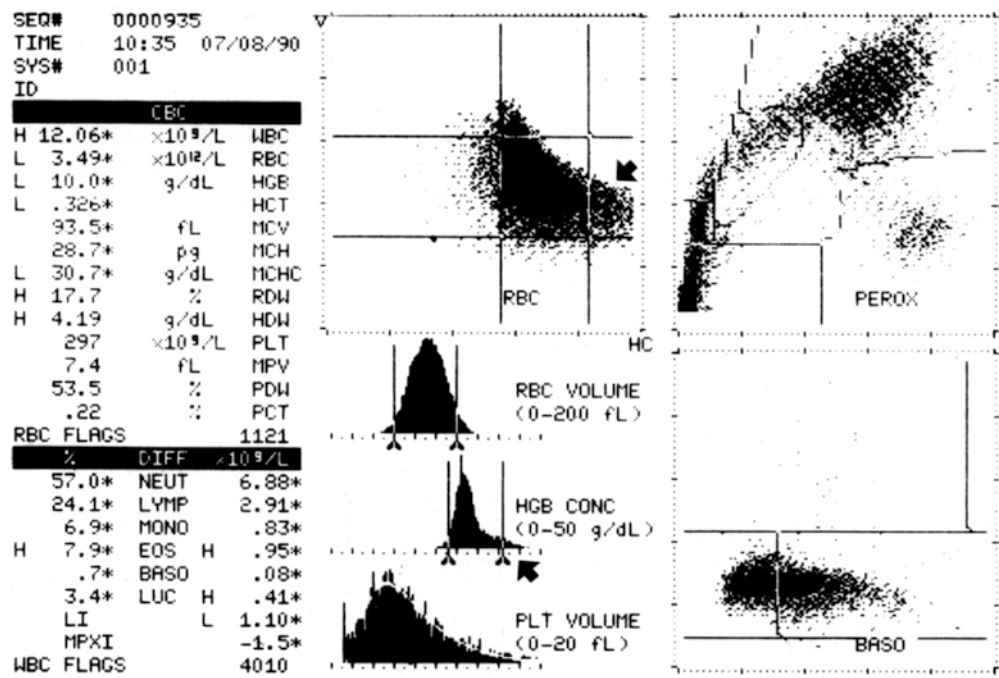


Fig. 4.20 Red cell cytogram and histograms from a Technicon H2 instrument showing an increase of hyperchromic cells (arrows), representing irreversibly sickled cells, and increased hypochromic macrocytes, representing reticulocytes.

Particularly high platelet counts are likely to be seen in the 7–8% of African Americans who are homozygous for *MPL*-Baltimore, a common polymorphism in the thrombopoietin receptor in this ethnic group [224].

Homozygosity for α +thalassaemia is associated with a higher RBC, Hb and haemoglobin A₂ percentage and a lower MCV, MCH, MCHC, reticulocyte count, irreversibly sickled cell count and haemoglobin F percentage, with heterozygotes having intermediate values [198]. Heterozygosity for α +thalassaemia and, even more so, homozygosity is associated with a significantly lower WBC [207]. The Hb is, on average, 10–20 g/l higher [114, 225]. The percentage of hyperdense cells is reduced. Patients with sickle cell anaemia with a high haemoglobin F percentage tend to have a higher Hb and MCV and a lower percentage of hyperdense cells. Those with the highest F levels (e.g. patients with the Saudi/Indian haplotype), also have a lower reticulocyte count.

Coexisting iron deficiency leads to a lower Hb, MCV, MCH and MCHC, although it is difficult to diagnose as the ferritin is often elevated by the ongoing inflammation that is present in all patients with sickle cell anaemia. There is an associated amelioration of haemolysis [201]. Patients who are maintained on folic acid have, on average, an MCV 4 fl lower than patients not so maintained [226]. A high MCV is usually due to administration of hydroxycarbamide, which may not be known to the laboratory. However, the possibility of coincidental vitamin B₁₂ deficiency must not be overlooked, particularly in patients who are being maintained on folic acid [227].

Changes occur in the blood count quite early in acute vaso-occlusive episodes. There is a fall in the Hb, a rise in the reticulocyte percentage and a rise in the MCHC, RDW, haemoglobin distribution width (HDW) and percentage of hyperdense cells [228]. The HDW is a measurement of the variation in haemoglobin concentration between individual red cells; its increase is a reflection of the increased number of hyperdense cells. As the acute episode progresses there is a return of RDW, HDW and percentage of hyperdense cells towards baseline values; the

percentage of hyperdense cells may fall below baseline values, probably because the most dense, least deformable cells are being preferentially trapped in the spleen and destroyed. The WBC and the neutrophil count increase during painful episodes and the platelet count and size may also increase [229]. When acute chest syndrome is caused by pulmonary fat embolism there is leucocytosis and usually a marked fall in Hb and platelet count [230]. Irregularly contracted cells, including hemi-ghosts, can appear in quite significant numbers, being indicative of hypoxia [231]. In a study of 257 patients with sickle cell disease, 218 of whom had sickle cell anaemia, presenting with acute chest syndrome, a baseline Hb above 82 g/l and a platelet count of above 440×10^9 /l were found to be predictive of pulmonary artery thrombosis when combined with a low PaCO₂ and the absence of a precipitating factor [232]. In addition to an acute fall in the Hb, splenic sequestration is associated with a fall in the platelet count, reticulocytosis and sometimes normoblastaemia [149].

Patients whose sickle cell anaemia is treated with hydroxycarbamide with a resultant increase in the haemoglobin F percentage show characteristic changes in the Hb and red cell indices. The Hb and the MCV rise while the MCHC, percentage of dense cells and reticulocyte count fall. The WBC, neutrophil count and platelet count can fall as a consequence of the cytotoxic effect of hydroxycarbamide and the associated reduction in inflammation and cellular adhesion.

Some newer treatments are starting to emerge and influence the phenotype of the disease. The majority of patients treated with voxelotor have a marked rise in Hb, to 100 g/l by 72 weeks in one study [233], together with a fall in the bilirubin concentration and the reticulocyte count and an increased oxygen affinity. Patients treated with the pyruvate kinase activator, mitapivat, similarly have a rise in the Hb, reduction of haemolysis and increased oxygen affinity [234]. It should be noted that an increase in oxygen affinity reduces oxygen delivery to tissues so that there may be no improvement in vaso-occlusive crises [235]. Other drugs, such as crizanlizumab and L-glutamine, have little or no effect on haematological parameters.

Blood film

The blood film is usually normal at birth and in the early neonatal period since the haemoglobin S percentage is relatively low, but this is not necessarily so (Fig. 4.21). Abnormalities are

usually detectable around six months of age (Fig. 4.22) when occasional sickle cells, target cells and Howell-Jolly bodies start to appear [208]. The majority of infants have features of hyposplenism by one year of age [208] and circulating erythroblasts, sickle

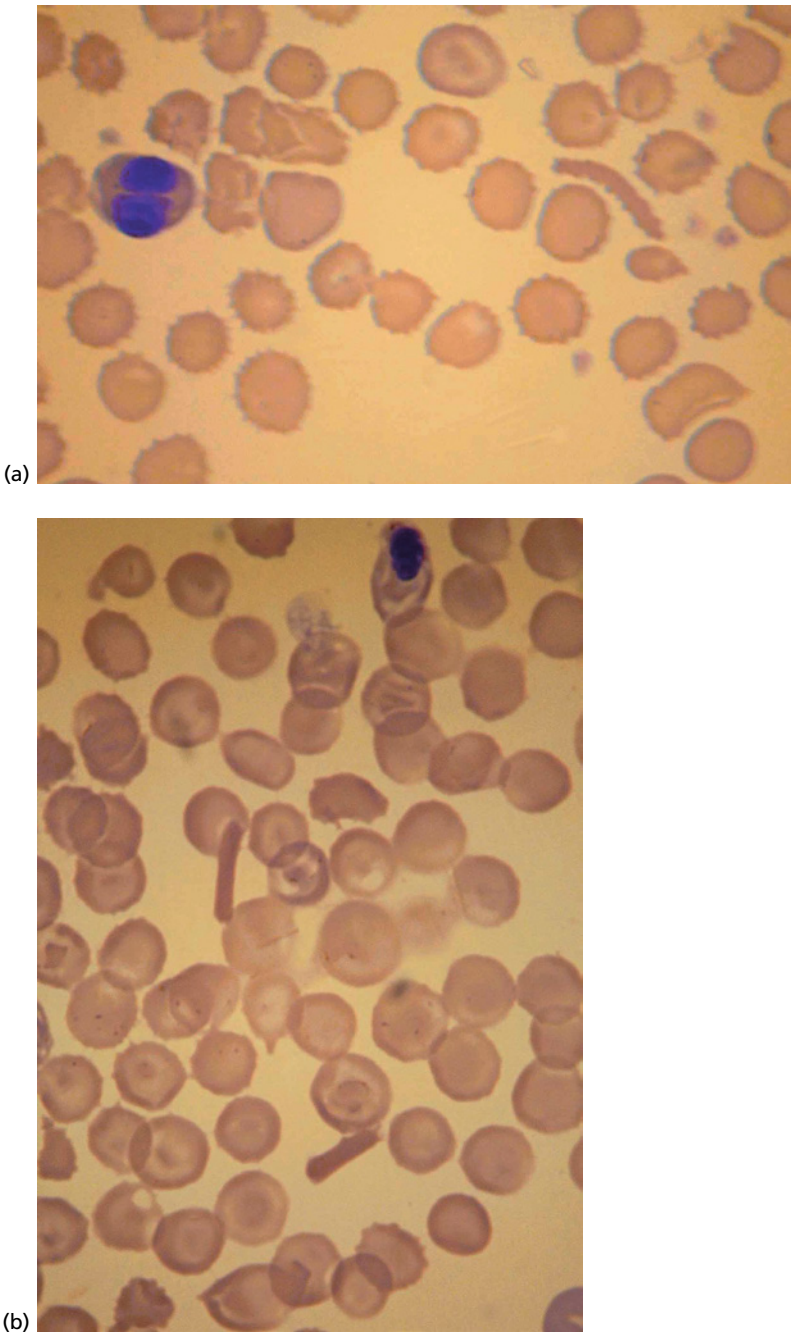


Fig. 4.21 Blood film of a neonate with sickle cell anaemia showing: (a) one sickle cell; (b) other poikilocytes consistent with reversibly sickled cells. MGG $\times 100$.

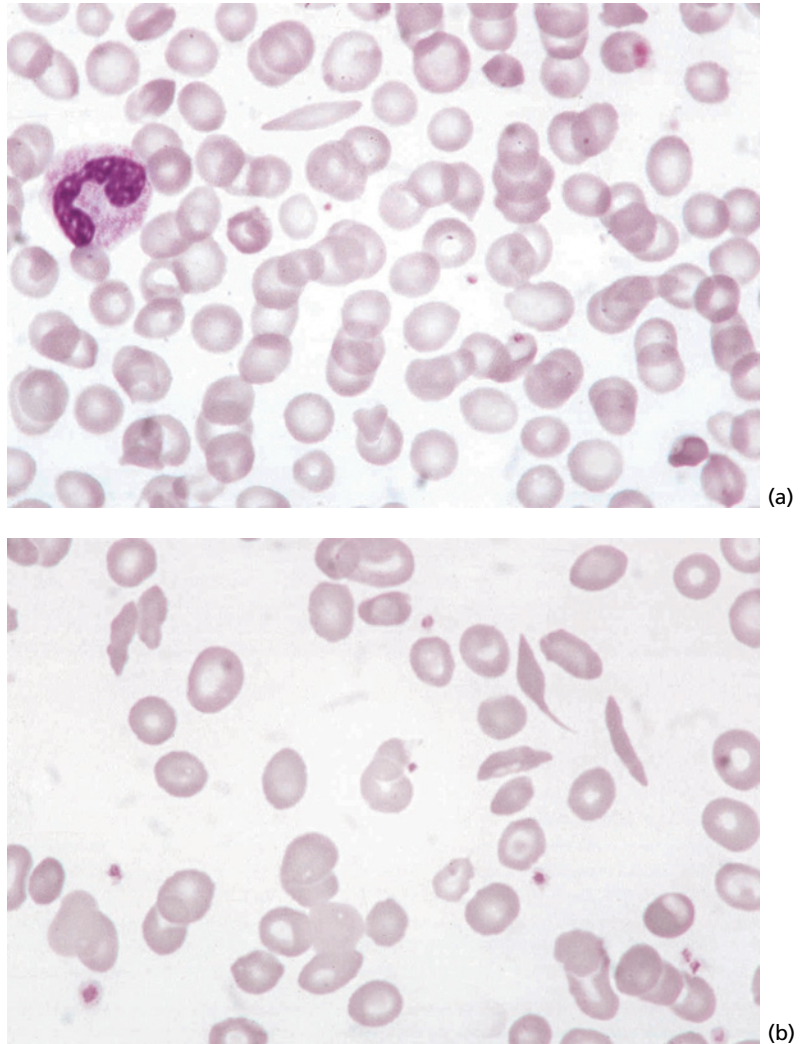


Fig. 4.22 The blood film of a child with sickle cell anaemia: (a) at the age of five months, showing mild anisopoikilocytosis and one sickle cell; (b) at the age of 13 years, showing more marked anisocytosis and sickle cell formation. MGG $\times 100$.

cells and Howell–Jolly bodies are much more common thereafter. In an adult with sickle cell anaemia, the blood film shows a variable number of crescent or sickle-shaped sickle cells (Fig. 4.23a). These represent irreversibly sickled cells that have not corrected their shape on exposure to atmospheric oxygen. The number of sickle cells is very variable, ranging from only occasional cells to 30–40%, and is partly determined by the age and condition of the blood sample before the blood film was made. They are less numerous in those with a lower MCHC [13]. In addition to classic sickle cells, there are elongated cells pointed at one or both

ends (Fig. 4.23b) [236]; these have been referred to as boat-shaped or oat-shaped cells or as plump sickle cells. The percentage of sickled cells plus boat-shaped cells increases with age, through childhood and adolescence, and correlates with severity of symptoms [237]. There is polychromasia and, in some patients, microcytosis and hypochromia. Small numbers of irregularly contracted cells may be seen (Fig. 4.23c) and sometimes there are cells in which the haemoglobin appears to have retracted into one half of the cell ('hemi-ghosts' or 'blister cells'); both these features are particularly common in patients with

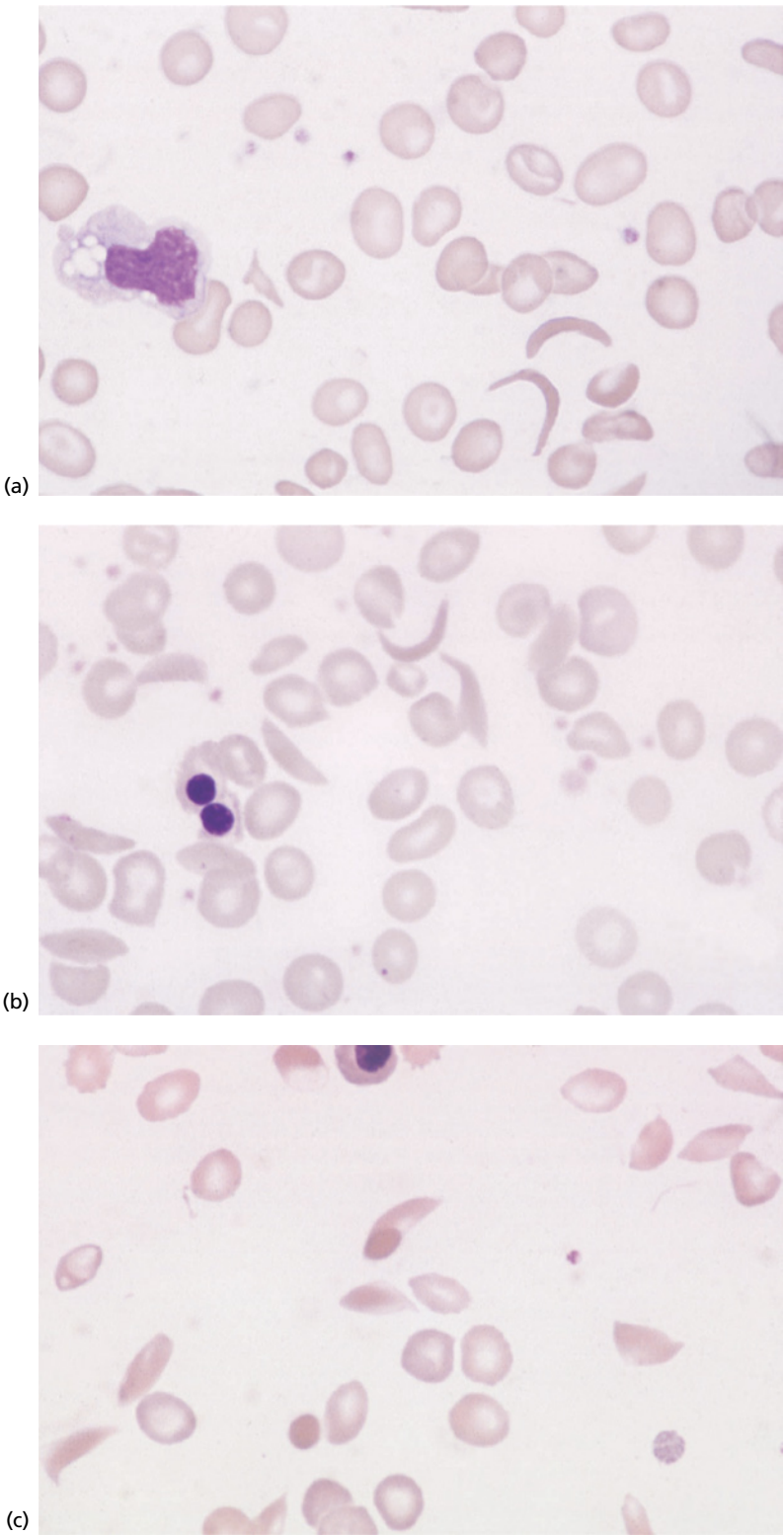


Fig. 4.23 Blood films of four patients with sickle cell anaemia showing the range of abnormality observed: (a) sickle cells and other poikilocytes; (b) sickle cells, a boat-shaped cell and nucleated red blood cells; (c) blood film during sickle cell crisis with pulmonary infarction and severe hypoxia showing one sickle cell, irregularly contracted cells, a hemi-ghost and several linear fragments (detached spicules of sickle cells); (d) minimal sickling but features of hyposplenism – a Howell–Jolly body, a large platelet and a target cell. MGG $\times 100$. (Continued on p. 241.)

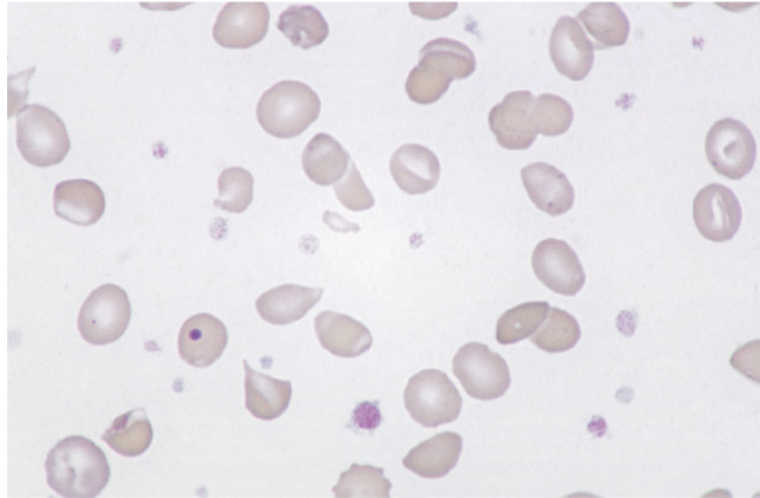


Fig. 4.23 *Continued.*

(d)

widespread pulmonary infarction and hypoxia. These abnormal red cells have increased density; their formation has been attributed to oxidant damage leading to transcellular bonding of damaged regions of the red cell membrane with trapping of haemoglobin within pseudovacuaes [238]. Linear red cell fragments may be present (see Fig. 4.23c); these were first described in a patient with cold agglutinins and a positive antiglobulin test [239] but in fact they are not rare, if specifically looked for. There are features of hyposplenism (Fig. 4.23d), specifically Howell–Jolly bodies, target cells, Pappenheimer bodies, an increased platelet count, increased platelet anisocytosis and sometimes an increased lymphocyte count. Acanthocytes, which are usually present in small numbers in hyposplenic individuals, are not usually a feature of hyposplenism in sickle cell disease. There are variable numbers of nucleated red blood cells (NRBC). The neutrophil count may be increased. Phagocytosis of erythrocytes by monocytes or neutrophils may be observed but is quite uncommon. Uncommonly in parvovirus B19 infection there is striking leucoerythroblastosis and thrombocytosis in advance of any reticulocyte response [240]. Following recovery from parvovirus-induced red cell aplasia, there is a rise in the reticulocyte count; there can also be an outpouring of erythroblasts into the peripheral blood, which can show

dyserythropoietic features as a result of ‘stress erythropoiesis’ [241] (Fig. 4.24).

In patients with sickle cell anaemia with a high haemoglobin F the abnormalities in the blood film are much less (Fig. 4.25). Sickle cells are less frequent and polychromasia and anaemia are less. The onset of features of hyposplenism is delayed. Coexisting α thalassaemia trait has also been observed to protect against the loss of splenic function [165, 242] although, surprisingly, in this study hyposplenism was not found to be related to age or haemoglobin F concentration [242]. Coexisting α thalassaemia trait (particularly homozygous α^0 thalassaemia trait) is associated with a blood film showing more target cells but fewer sickle cells [13].

Therapy with hydroxycarbamide leads to macrocytosis, a reduction in the number of sickle cells and boat-shaped cells and lessening of polychromasia. Therapy with voxelotor, which increases the oxygen affinity of haemoglobin S, leads to a rise in the Hb and a reduction in the number of sickle cells.

During acute vaso-occlusive complications there is a slight worsening of anaemia. There may be a further elevation of the neutrophil count, left shift and an increase in the numbers of NRBC. An increase in the number of sickle cells, in comparison with the same individual’s blood film in the steady state, has been reported but not all investigators confirm this observation [243]. An increase in the number of

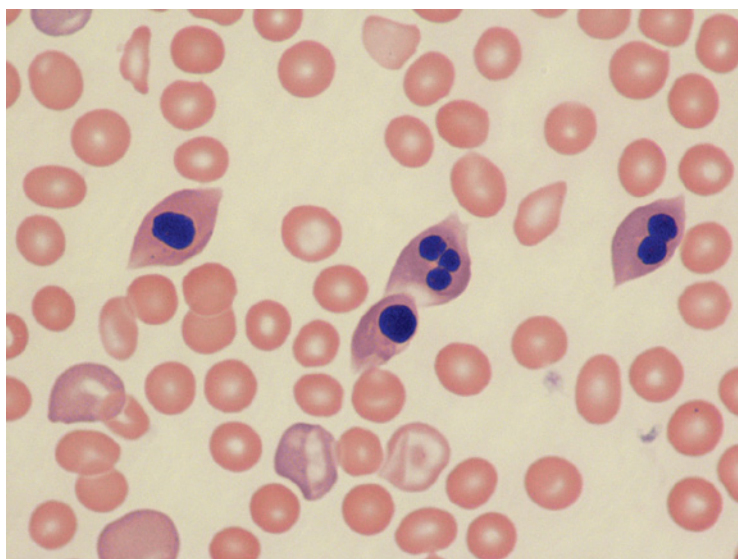


Fig. 4.24 Blood film showing dyserythropoiesis as a feature of 'stress erythropoiesis' during recovery from an aplastic crisis. MGG $\times 100$.

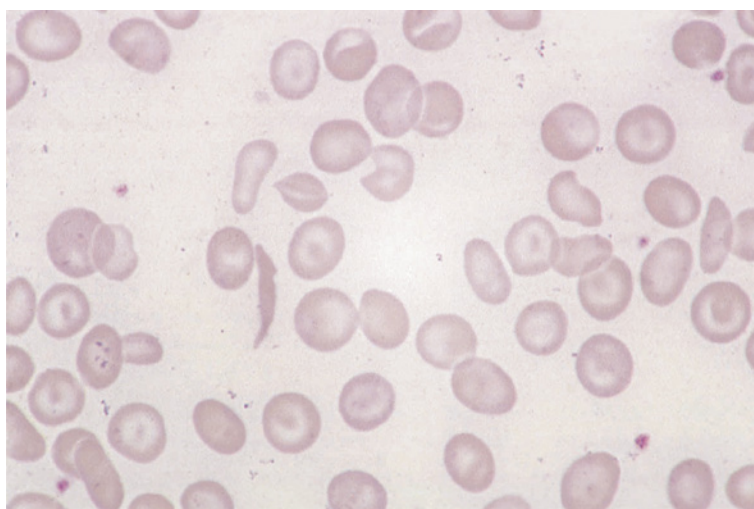


Fig. 4.25 Blood film of an Arab patient with a high haemoglobin F percentage and clinically mild disease; there were numerous target cells but only occasional sickle cells. MGG $\times 100$.

spiculated or echinocytic sickle cells has also been noted [243]. Irregularly contracted cells 'hemi-ghosts' can increase in number, particularly in those with severe hypoxia and extensive sickling within the pulmonary vasculature (see Fig. 4.23c). In one scanning electron microscopy study, acute vaso-occlusive complications were associated with the presence of echinocytes, echinocytic sickle cells, 'blister cells' and macrocytes but there was no increase in the number of non-echinocytic irreversibly sickled cells [244]. When there is extensive bone marrow infarction there is a greater fall in the

Hb and platelet count together with the appearance of increasing numbers of NRBCs.

Various other complications of sickle cell anaemia may be apparent from the full blood count (FBC) and the blood film. Parvovirus B19 infection may be suspected when there is worsening anaemia with a lack of polychromasia. The platelet count is also often reduced. When recovery occurs there is initially the appearance of numerous NRBCs in the peripheral blood followed by reticulocytosis and thrombocytosis. In splenic sequestration there is an acute fall in the Hb and sickle cells may be relatively

infrequent; subsequently there is reticulocytosis with increasing numbers of NRBCs. The platelet count may also be reduced. In chronic hypersplenism there is increased anaemia, thrombocytopenia and reticulocytosis. When there is complicating megaloblastic anaemia there may be macrocytes, oval macrocytes and hypersegmented neutrophils; polychromasia is less than would otherwise be expected in a patient with sickle cell anaemia. Patients with coexisting sickle cell anaemia and homozygosity for α^+ thalassaemia who develop megaloblastic anaemia may show an increase in the MCV and MCH but with both values remaining within the normal range rather than exceeding it. Because of the shortened red cell life span, megaloblastic anaemia can also have an acute onset with pancytopenia and a rapidly falling Hb without macrocytosis. In patients with a delayed transfusion reaction, some spherocytes are seen but it can be difficult to recognise the morphological features of a transfusion reaction in a patient with sickle cell anaemia. The direct antiglobulin test is positive. Patients with sickle cell disease sometimes develop severe haemolysis following blood transfusion, with both homologous and autologous cells being destroyed; some such cases have a negative direct antiglobulin test, low reticulocyte count and a high ferritin and may be attributable to erythrophagocytosis by activated macrophages [245]. During intercurrent infections the patient with sickle cell anaemia is likely to show neutrophilia, left shift, toxic granulation and sometimes an increase in the platelet count; rarely organisms (e.g. pneumococci) are found within neutrophils.

Other tests

In the adult, haemoglobin electrophoresis, HPLC and capillary electrophoresis show haemoglobins S, F and A_2 (Figs 4.26–4.28). Haemoglobin S is the major haemoglobin present, usually comprising 90–95% of total haemoglobin, although measurements are typically significantly less due to inconsistencies in quantifying post-translationally modified haemoglobins. Haemoglobin A is totally absent.

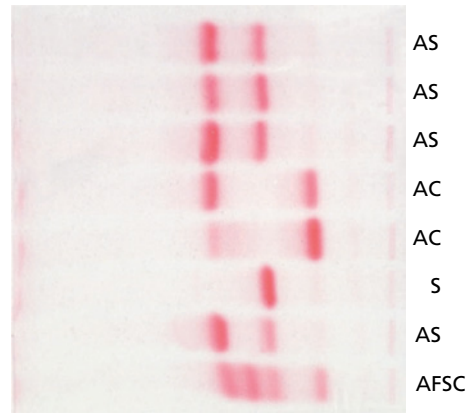


Fig. 4.26 Haemoglobin electrophoresis on cellulose acetate at alkaline pH showing a patient with sickle cell anaemia (third from bottom) with almost all the haemoglobin being haemoglobin S; AFSC indicates a control sample containing haemoglobins A, F, S and C, while other lanes show patients with either sickle cell trait (AS) or haemoglobin C trait (AC).

Haemoglobin $A_{2\gamma}$, which inhibits the polymerisation of haemoglobin S, can be present in normal amounts or be slightly elevated (usually 2–4%) [13, 198, 246]. The level is influenced by polymorphism in *BCL11A* and the *HBSIL-MYB* interval and shows an inverse relationship with haemoglobin F percentage [246]. Higher percentages are seen in those who also have α thalassaemia trait, although there is considerable variation in the mean levels reported in different series of patients (Table 4.8) [13, 202, 247–250]. Haemoglobin F is usually only slightly elevated (see later). Inevitably, the percentage of haemoglobin S correlates inversely with the percentage of haemoglobin F. The percentage of haemoglobin S also varies with the number of α genes and with the MCV and MCH. Looked at in another way, the coexistence of α thalassaemia trait with sickle cell anaemia leads to reduction of the MCV and MCH and a slight reduction of haemoglobin S percentage. Free α globin chain is increased; this correlates with a higher lactate dehydrogenase (LDH), bilirubin, dense red cell percentage, MCV, MCH and reticulocyte % and inversely with haemoglobin A_2 percentage [251].

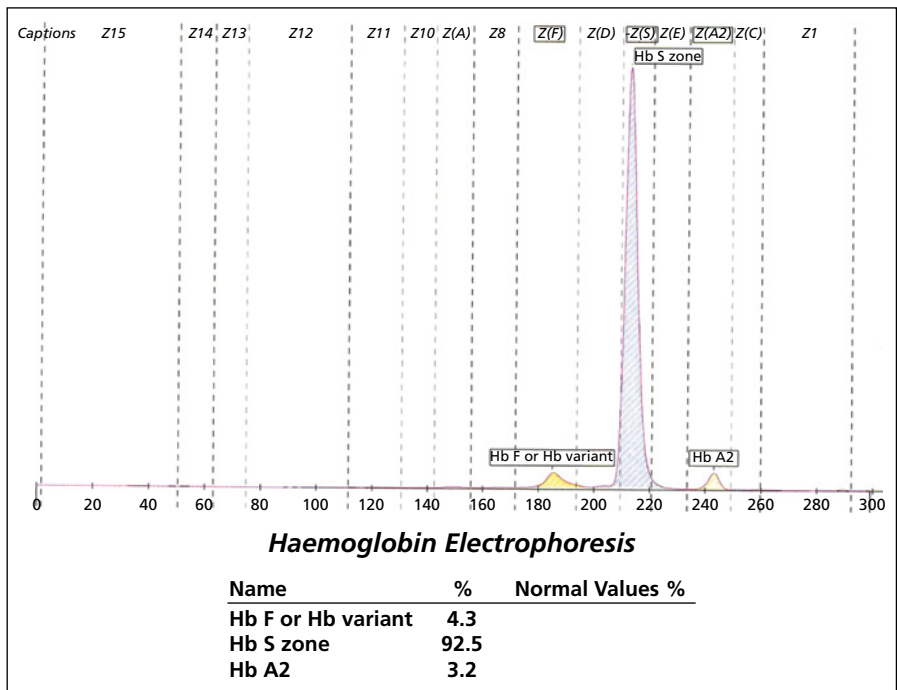


Fig. 4.27 Capillary electrophoresis in sickle cell anaemia (Sebia CapillaryS 3) showing haemoglobins F, S and A₂. Haemoglobin F is slightly increased and haemoglobin A₂ is normal.

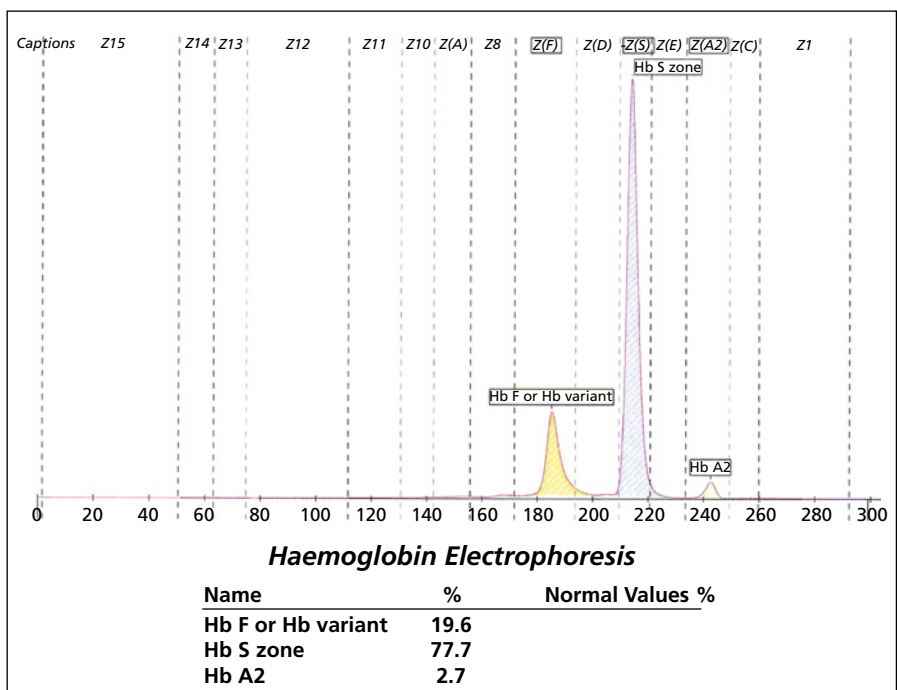


Fig. 4.28 Capillary electrophoresis in sickle cell anaemia (Sebia CapillaryS 3) showing haemoglobins F, S and A₂. Haemoglobin F is moderately increased as a result of hydroxycarbamide therapy while haemoglobin A₂ is normal.

Table 4.8 Haematological characteristics and percentages of various haemoglobins in adults with sickle cell anaemia and other conditions with haemoglobins S, F and A₂ only. (Derived from references [13, 202, 247–250] and other sources.)

Genotype	Usual Hb (g/l)	Usual MCV (fl)	Usual Reticulocyte count (%)	Usual Hb F percentage	Usual Hb A ₂ percentage
SS	60–100	70–100	5–20	Usually 5–10 but up to 40*	1.6–3.6† (occasionally up to 5%)‡ 2.4–4.9§
Sβ ⁰	70–110	60–80	8–9	5–15	4–5.6† 4.0–5.3§
S/δβ ⁰	100–120	76–83	2–4	15–25	1.9–2.3
S/HPFH	>120	68–88¶	Normal	20–30	1.1–2.2 in the majority

Hb, haemoglobin concentration; HPFH, hereditary persistence of fetal haemoglobin; MCV, mean cell volume.

* Influenced by coinheritance of non-deletional HPFH as well as by the haplotype associated with the β^s gene [248, 249].

Saudi-Indian haplotype, 10–25% Hb F

Senegal haplotype, 7–10% Hb F

Benin or Bantu haplotype, 6–7% Hb F

Cameroon haplotype, 5–6% Hb F

Mean (and SD) of 6.06 (± 4.23) for 120 SS adults in the UK [247].

† Some overlap occurs, particularly when coexisting homozygous α⁺ thalassaemia raises the A₂ percentage in cases of SS [13]; in one series reported mean A₂ levels were 2.8% with four α genes, 3.3% with three α genes and 3.8% with two α genes [248]; in another series reported levels were higher – 3.5%, 3.7% and 4.9% respectively [202].

‡ High levels are characteristically seen in the Arab-Indian mutation.

§ When measured by capillary electrophoresis [250].

¶ Normal if there is no coexisting α thalassaemia trait.

The sickle solubility test is positive and immunoassays [252] demonstrate the presence of haemoglobin S with no haemoglobin A.

Neonates with sickle cell anaemia usually have predominantly haemoglobin F with haemoglobin S comprising only a low percentage of total haemoglobin (Fig 4.29). Sometimes only haemoglobin F is present and DNA analysis or repeat testing when the baby is a few months of age is then necessary for diagnosis. In the neonatal period, diagnostic confusion can occur not only with sickle cell/β⁰ thalassaemia but also with sickle cell/β⁺ thalassaemia and sickle cell/hereditary persistence of fetal haemoglobin (HPFH) [253] (see later). The post-natal fall in haemoglobin F is slower in babies with sickle cell anaemia than in haematologically normal babies with mean levels of about 20% at one year of age [208].

The oxygen dissociation curve shows reduced oxygen affinity (i.e. a right-shifted curve and an increased P₅₀) [254]. The right shift is less in

those with a high haemoglobin F percentage, either as a feature of the disease or as a result of hydroxycarbamide therapy. Resting arterial oxygen saturation in the steady state is usually greater than 95% but in patients with significant pulmonary damage may be reduced (e.g. to 80–95%).

Studies of globin chain synthesis show balanced synthesis of α and β^s globin chains unless there is coexisting α thalassaemia trait.

Bilirubin concentration is increased, the bilirubin being mainly unconjugated. LDH is increased approximately twofold; high levels correlate with other evidence of haemolysis and with end organ vasculopathy, probably because a greater degree of haemolysis is associated with vascular endothelial dysfunction [255]. Hyperuricaemia is common. Serum haptoglobin is usually absent and Schumm test for methaemalbumin can be positive. Red cell survival studies show a half-life (T_{1/2}) of about 7–14 days, less if there is splenomegaly.

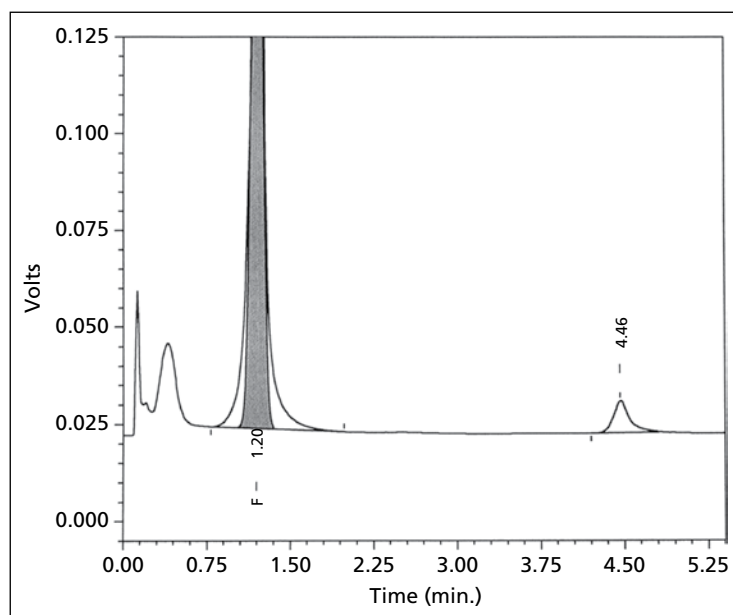


Fig. 4.29 HPLC chromatogram (Bio-Rad Variant II) in a neonate with sickle cell anaemia showing mainly haemoglobin F₀ (dark grey), total absence of haemoglobin A and presence of haemoglobin S; haemoglobin S was 5.5% and the retention time was 4.46 minutes; the peaks to the left of haemoglobin F₀ represent post-translationally modified haemoglobin F.

Heterozygous α^+ thalassaemia is associated with longer red cell survival. Coexisting iron deficiency leads to a considerable improvement in red cell survival, associated with a fall in bilirubin concentration and LDH [201]. Hydroxycarbamide therapy also leads to improved red cell survival and reduced biochemical evidence of haemolysis [256].

A bone marrow aspirate shows erythroid hyperplasia and the presence of sickle cells (Fig. 4.30). Dyserythropoiesis can be present. Macrophages are increased and may contain sickled cells (Fig. 4.31). Vaso-occlusive episodes can be complicated by haemophagocytic lymphohistiocytosis and the bone marrow then shows haemophagocytosis [257]. As a result of increased cell turnover, foamy macrophages and sea-blue histiocytes can be increased and pseudo-Gaucher cells are sometimes numerous [258] (Fig. 4.32). A trephine biopsy section likewise shows erythroid hyperplasia and sickle cells inside macrophages and within blood vessels (Fig. 4.33).

Haemoglobin F percentage

In sickle cell anaemia, haemoglobin F is usually around 5–10% but may be higher, sometimes comprising up to 40% of total haemoglobin (see

Table 4.8). The level is higher in infancy and tends to be higher in women than in men [219]. The switch from γ to β^S synthesis is delayed compared with the γ to β switch in normal subjects. The percentage of haemoglobin F falls most rapidly in the first three years of life and then more slowly with increasing age; by the age of 10 years haemoglobin F levels approximate those in adult life although they continue to decrease slowly throughout life. The percentage of haemoglobin F in an individual is determined by factors related and unrelated to the β globin gene cluster, although less than 50% of the variation seen in haemoglobin F levels can be explained by known genetic factors. There is a clear relationship to the haplotype associated with the β^S gene, averaging 10%, 7%, 7% and 5% respectively in the Senegal, Benin, Cameroon and Bantu haplotypes and 15–23% in the Arab-Indian haplotype [259]. In other studies, the Senegal haplotype was associated with a haemoglobin F level of 7–10% in adults whereas adults with the Bantu and Benin haplotypes averaged around 6–7%, but with a wide range; the Cameroon haplotype had haemoglobin F averaging 5–6% [248, 249]. Similarly, in two further studies, the Arab-Indian haplotype was associated with a haemoglobin F level of

Fig. 4.30 Bone marrow aspirate in sickle cell anaemia showing erythroid hyperplasia and two sickle cells. MGG $\times 100$.

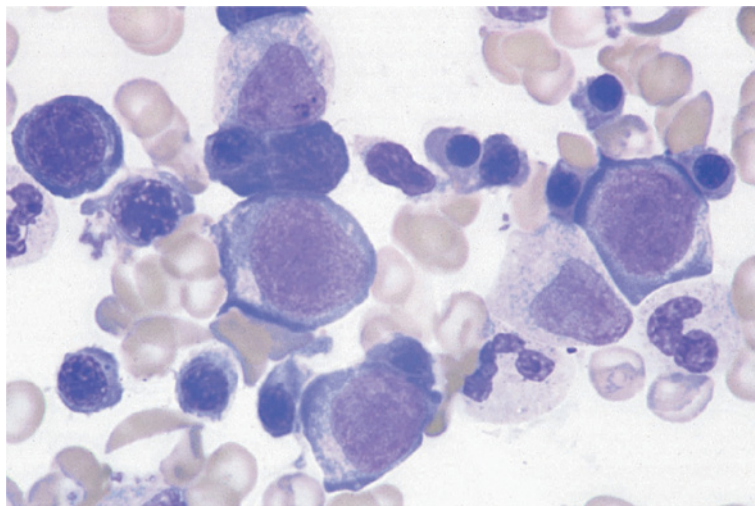


Fig. 4.31 Bone marrow aspirate in sickle cell anaemia showing a sea-blue histiocyte packed with sickle cells. MGG $\times 100$.

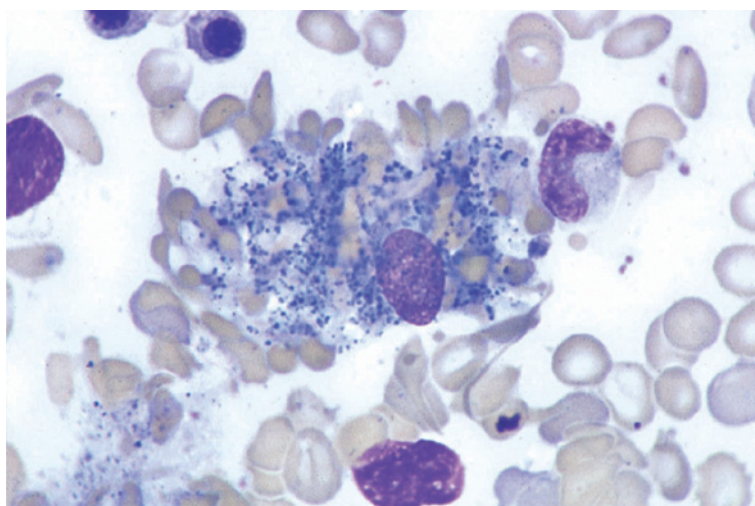
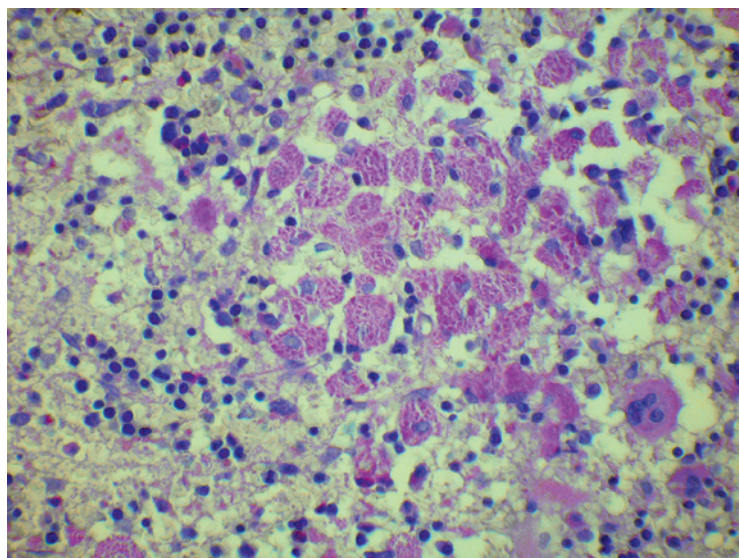


Fig. 4.32 Trephine biopsy section in sickle cell anaemia showing numerous pseudo-Gaucher cells. Periodic acid-Schiff stain $\times 40$.



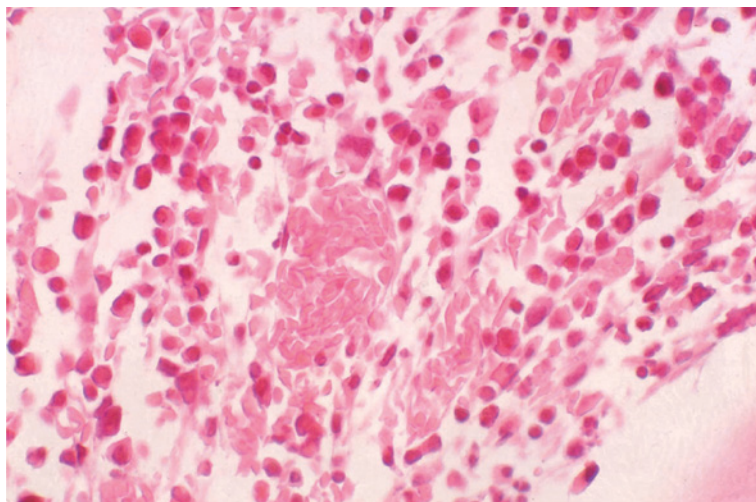


Fig. 4.33 Trephine biopsy section in sickle cell anaemia showing erythroid hyperplasia and two vessels packed with sickle cells. Haematoxylin and eosin $\times 40$.

10–25% [249] and 18–41% [131] respectively. Saudi Arabs who have African haplotypes (74% Benin, 22% Bantu) have a haemoglobin F about twice that of African Americans [131]. Arabs with the Senegal haplotype also have a higher haemoglobin F percentage than individuals of African ancestry [259].

The relationship of haplotype to haemoglobin F percentage appears to result from an association between haplotype and determinants of non-deletional HPFH. Both the Arab-Indian haplotype and the Senegal haplotype are linked with the common $-158 \text{ }^{\text{C}}\gamma \text{ C} \rightarrow \text{T}$ variant [260] whereas the Benin haplotype, which has a low percentage of haemoglobin F, is not linked with $-158 \text{ }^{\text{C}}\gamma \text{ C} \rightarrow \text{T}$. The Senegal haplotype is also associated with a variant in the promoter of *HBG2* [137]. A variant at $^{\text{A}}\gamma \text{ IVS-II}$ has also been linked to the high haemoglobin F level observed when the β^{s} gene is associated with the Senegal and Arab-Indian haplotypes [261]. In addition, the Arab-Indian haplotype is associated with a variant at -530 bp (where there is $(\text{AT})_9\text{T}_5$ rather than $(\text{AT})_7\text{T}_7$, causing increased affinity for a BP-1 (a negative *trans*-acting factor) and repression of β^{s} synthesis). The higher haemoglobin F in sickle cell anaemia associated with the Arab-Indian haplotype, in comparison with that in the Senegal haplotype, may be related to the combined effect of the $(\text{AT})_x(\text{T})_y$ variant and the $-158 \text{ }^{\text{C}}\gamma \text{ C} \rightarrow \text{T}$ and $^{\text{A}}\gamma \text{ IVS-II}$ variants. The

$^{\text{C}}\gamma\text{:}^{\text{A}}\gamma$ ratio is increased in those with a high haemoglobin F in association with the Saudi Arabian or Senegal haplotype [243] while the $\text{G}\gamma$ promoter associated with the Bantu haplotype has been shown to be associated with low $\text{G}\gamma$ synthesis [262]. In Saudi but not Indian subjects, increased haemoglobin F is partly related to polymorphism of the *ANTXR1* gene acting in *trans* [263]. Increased haemoglobin F in Brazilian patients, associated with a reduced rate of sickle-related complications, has been linked to enhancer polymorphisms in *BCL11A* and *MYB* [264]. Increased expression of *BCL2L1* is associated with a higher haemoglobin F percentage [265]. Polymorphisms in the *HBS1L-MYB* intergenic region also contribute. The effect of *BCL11A* expression on haemoglobin F has been used in gene editing to enhance haemoglobin F expression and ameliorate the condition [266].

The percentage of F cells (i.e. of cells containing haemoglobin F) is increased in sickle cell anaemia. In one study the mean count was 55% (range 17–94%), the normal level being 0.5–7% [267]; the log of the haemoglobin F concentration correlated with the percentage of F cells. In another study the X-linked F-cell production locus was found to be the major determinant of haemoglobin F percentage in patients with sickle cell anaemia in association with the three major African haplotypes [268]; factors linked to

the β gene haplotype were next most important. The effect of the X-linked F-cell locus may be the reason that women with sickle cell anaemia, like haematologically normal women, tend to have a higher haemoglobin F level than men.

Individuals with coexisting α thalassaemia trait have been observed to have significantly higher proportion of haemoglobin F in the first decade of life [225] but thereafter have a somewhat lower proportion than those with four α genes [198, 269].

The haemoglobin F percentage in sickle cell anaemia is of prognostic significance [193], the prognosis being more favourable when the percentage is higher. The haemoglobin F percentage is increased 2–16 fold by hydroxycarbamide therapy [256].

Diagnosis

Diagnosis rests on the demonstration of haemoglobins S, F and A_2 only with the presence of haemoglobin S as the sole β chain variant haemoglobin being confirmed by at least two independent techniques. In patients with microcytosis or with a significant increase of haemoglobin F, the possibility of compound heterozygosity for S and β^0 or $\delta\beta^0$ thalassaemia or S and deletional HPFH respectively must be considered before a diagnosis of sickle cell anaemia is made.

When haemoglobin electrophoresis is the primary technique, it is important not to misdiagnose compound heterozygous states for S and D, G, Korle Bu or Lepore as sickle cell anaemia. Recognising the presence of D, G, Korle Bu or Lepore in compound heterozygous states with haemoglobin S is more complex than recognising the simple heterozygous state since all of these have a single band on cellulose acetate electrophoresis and a positive sickle solubility test (whereas the simple heterozygous states for any of these variant haemoglobins may simulate sickle cell trait on electrophoresis at alkaline pH but are easily distinguished since the sickle solubility test is negative). DNA analysis is increasingly used to confirm the diagnosis of sickle cell anaemia, particularly when the results of family studies or antenatal screening

are not available or are inconsistent. DNA analysis can also identify coexistent α thalassaemia and can potentially be used for extended blood group analysis to facilitate the selection of blood for future transfusions.

Effects of treatment

Treatment with hydroxycarbamide increases both the percentage and absolute amount of haemoglobin F.

Treatment with voxelotor alters the physico-chemical properties of haemoglobins, binding to α chains and increasing oxygen affinity. It thus alters the characteristics of haemoglobins A, A_2 and F, which can lead to either double peaks or two distinct peaks on both HPLC (Fig. 4.34a) and capillary electrophoresis (Fig. 4.34b) [270, 271]. Whether there are two distinct peaks or overlapping peaks of S and altered S on HPLC can be instrument dependent, overlapping being seen, for example, with the Bio-Rad Variant II Beta Thalassaemia programme but not with the Bio-Rad Variant II Dual Programme (which also measure haemoglobin A1c). Extra bands are also apparent on isoelectric focusing [270].

Interactions of haemoglobin S homozygosity with other thalassaemias, haemoglobinopathies and other inherited erythrocyte abnormalities

The modification of sickle cell anaemia by coinheritance of α thalassaemia trait or non-deletional HPFH has been discussed earlier.

Coinheritance of certain α chain variants, including haemoglobin Korle Bu, haemoglobin Memphis and haemoglobin Hopkins II, ameliorates sickle cell anaemia.

Coinheritance of other α chain variants, for example haemoglobin G-Philadelphia and haemoglobin Stanleyville II, has no significant effect on the clinical or haematological features of sickle cell anaemia [272, 273]. The results of haemoglobin electrophoresis and HPLC may be complex. With sickle cell anaemia and haemoglobin G-Philadelphia there are two bands, an S band and a G-Philadelphia/S hybrid band

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.3	0.63	3180
Unknown	---	7.4	1.05	69203
F	30.6*	---	1.14	270794
Ao	---	3.4	2.25	31401
A2	3.0	---	3.67	27340
Unknown	---	8.1	4.22	74801
S-window	---	48.7	4.40	452420

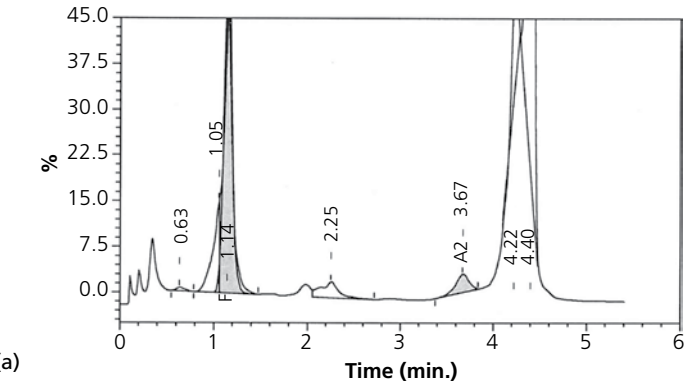
Total Area: 929,138*

F Concentration = 30.6* %

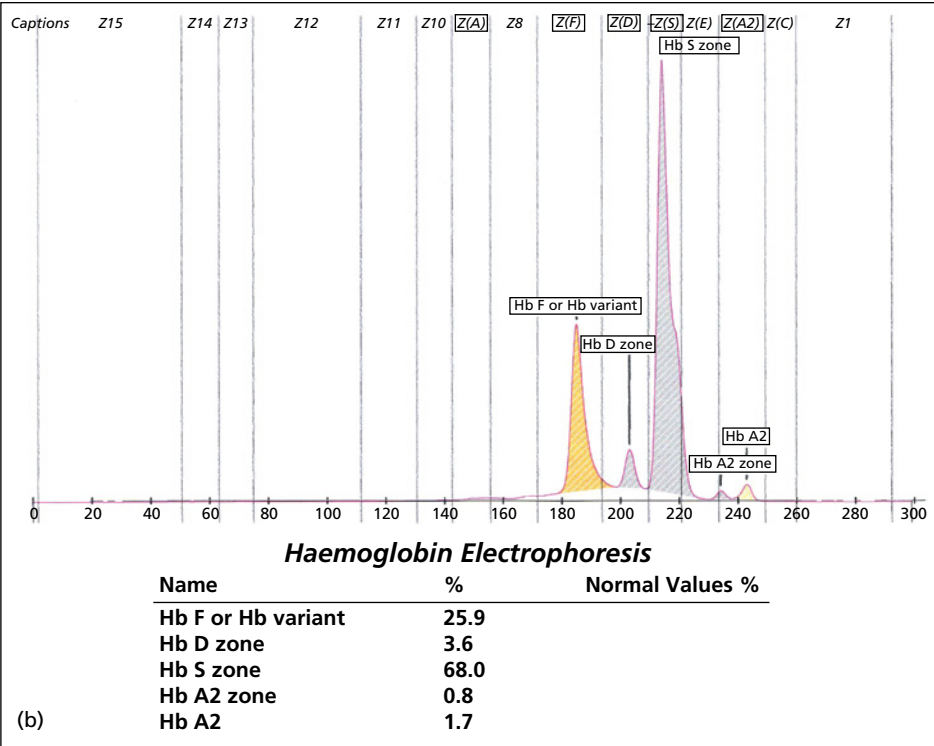
A2 Concentration = 3.0 %

*Values outside of expected ranges

Analysis comments:



(a)



(b)

Fig. 4.34 HPLC and capillary electrophoresis in a patient with sickle cell anaemia being treated with voxelotor: (a) HPLC (Bio-Rad Variant II) showing double peaks for both haemoglobin F and haemoglobin S; (b) capillary electrophoresis (Sebia Capillarys) showing double peaks for haemoglobins F and A₂ with the shape of the haemoglobin S peak being altered by the presence of the altered haemoglobin.

which has the same mobility on alkaline pH as haemoglobin C (Fig. 4.35a,b). The proportion of haemoglobin S is greater than the proportion of the hybrid band [272]. At acid pH there is a single band with the mobility of S, since at this pH the hybrid has the same mobility as S. Coinheritance with the α chain variant, Hb Montgomery also produces a hybrid band that has characteristics resembling those of haemoglobin C on both cellulose acetate electrophoresis and HPLC [274]. HPLC can show not only hybrid peaks but also, in the case of an α chain variant, a haemoglobin A₂ variant.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is common in many of the ethnic groups with a high prevalence of the β^S allele. Coinheritance of G6PD deficiency is associated with a lowering of Hb on average by 10 g/l [199, 275], with infants showing a higher reticulocyte count [275] and a higher rate of acute anaemic events [275]; effects lessen after two years of age, probably because a fall of haemoglobin F percentage is associated with a higher reticulocyte count and therefore a higher concentration of G6PD [275]. In adults, a study from France found that although the concentration of

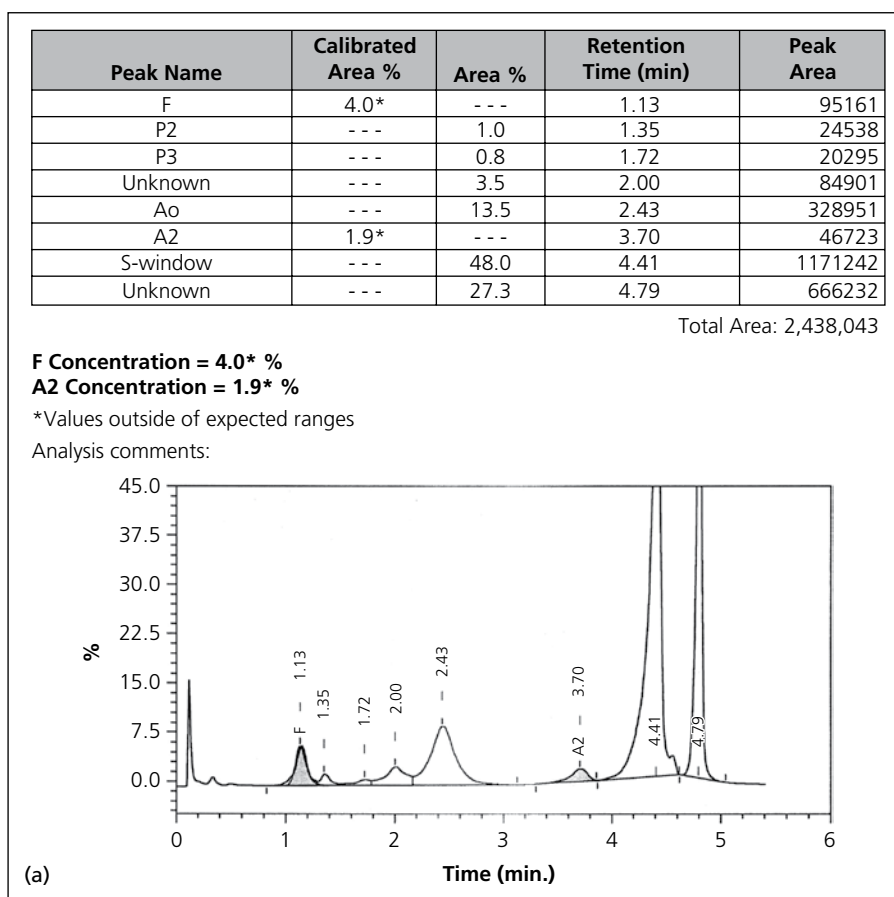


Fig. 4.35 HPLC and capillary electrophoresis in a patient with sickle cell anaemia and heterozygosity for haemoglobin G-Philadelphia, who has been transfused showing: (a) on HPLC (Bio-Rad Variant II) the hybrid peak, $\alpha^C_2/\beta^S_{2\gamma}$, is 'unknown'; the post-translationally modified hybrid is responsible for the shoulder on the right of the haemoglobin S peak while the sharp peak far left is likely to represent bilirubin; (b) capillary electrophoresis (Sebia Capillarys) showing the hybrid haemoglobin in the C zone with an A₂ variant with a haemoglobin G α chain appearing as a low peak to the right. (Continued on p. 252.)

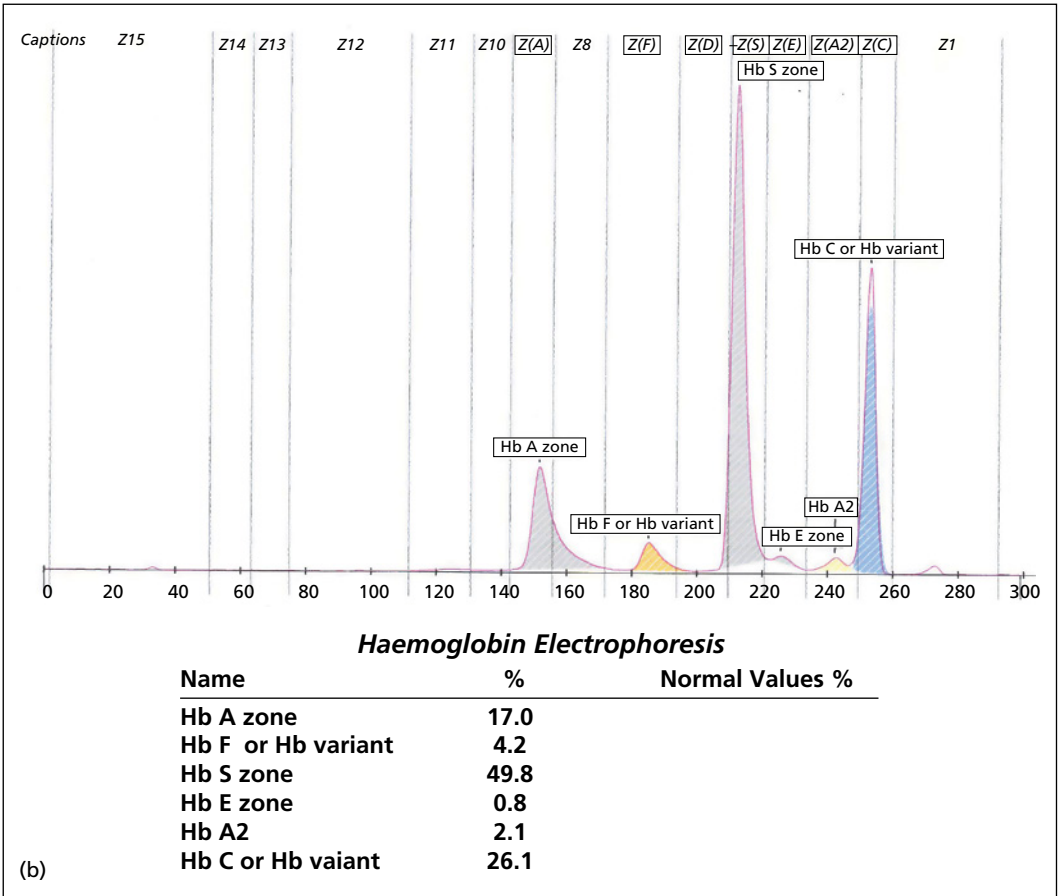


Fig. 4.35 Continued.

reactive oxygen species in red cells was higher in G6PD-deficient subjects, the Hb and clinical features did not differ [276]. Contrary results were found in a study, mainly in children, in the Democratic Republic of the Congo with transfusion requirement being higher and vaso-occlusive crises being more common in deficient subjects [277]. G6PD deficiency has also been variably associated with an increased risk of cerebrovascular disease in sickle cell anaemia.

Sickle cell/haemoglobin C disease

Sickle cell/haemoglobin C disease is consequent on coinheritance of β^S and β^C alleles. There is no normal β gene and therefore no haemoglobin A. This compound heterozygous state leads to a sickling disorder that is similar to sickle cell

anaemia but on average is somewhat less severe. Although most diagnoses are made in childhood or adolescence, some patients have few symptoms and, in the absence of neonatal screening programmes, around a quarter of cases are diagnosed in adult life [278]. The degree of haemolysis is less than in sickle cell anaemia with red cells surviving around 27–29 days, in comparison with around 15–17 days. Life expectancy is considerably better than that of sickle cell anaemia. In the USA the average survival reported in 1994 was 60 years for men and 68 years for women [193] and by 2014 median survival was estimated at 66 years [195]. Another US study in 2019 estimated median survival at 55 or 62 years, depending on the statistical method applied [139].

Sickle cell haemoglobin C disease is characterised by increased density of red cells, which is attributable to an increased K—Cl co-transport with loss of intracellular potassium and cellular dehydration [279]. This, in turn, increases the likelihood of polymerisation of haemoglobin S and, together with the higher haemoglobin S percentage (averaging 50% rather than 40%), helps to explain why the compound heterozygous state generally causes significant disease, whereas sickle cell trait does not [279].

Variant haemoglobins in which there is a second mutation in the β^c allele are likely to interact with haemoglobin S in a similar manner to haemoglobin C itself. One such haemoglobin is haemoglobin Arlington Park, $\beta^{6\text{Glu} \rightarrow \text{Lys}}, 95\text{Lys} \rightarrow \text{Glu}$, which will be missed on cellulose acetate electrophoresis at alkaline electrophoresis since there is no net charge change in comparison with haemoglobin A [280, 281].

Clinical features

Sickle cell/haemoglobin C disease leads to a chronic haemolytic anaemia and to intermittent

acute vaso-occlusive complications, similar to those of sickle cell anaemia but less frequent. Dactylitis is quite uncommon [282]. The Hb is higher than in sickle cell anaemia and the degree of haemolysis is less; the higher Hb is likely to be mainly due to a lesser reduction of the oxygen affinity rather than to less severe haemolysis. Aseptic necrosis (Fig. 4.36) and bone marrow infarction, with embolism of necrotic bone marrow to the lungs, are more common than in sickle cell anaemia. In one series of patients, 15% suffered osteonecrosis of femoral or humeral heads or vertebral bodies and two of 284 patients died of bone marrow embolism (2 of 25 deaths) [282]. Bone marrow necrosis and fat embolism syndrome is associated with bone pain and can lead to acute renal failure [283]. Retinal disease (retinitis proliferans and vitreous haemorrhages) is more frequent and more severe; in one series of patients it was seen in 21% and in another in 23% [282]. In a third series of 15 patients, retinal disease was seen in 34% of patients; retinopathy correlated with a higher Hb in this cohort [284] and in a fourth series of patients [285]. Iris atrophy as a result of ischaemia can also occur [286]. Sensorineural

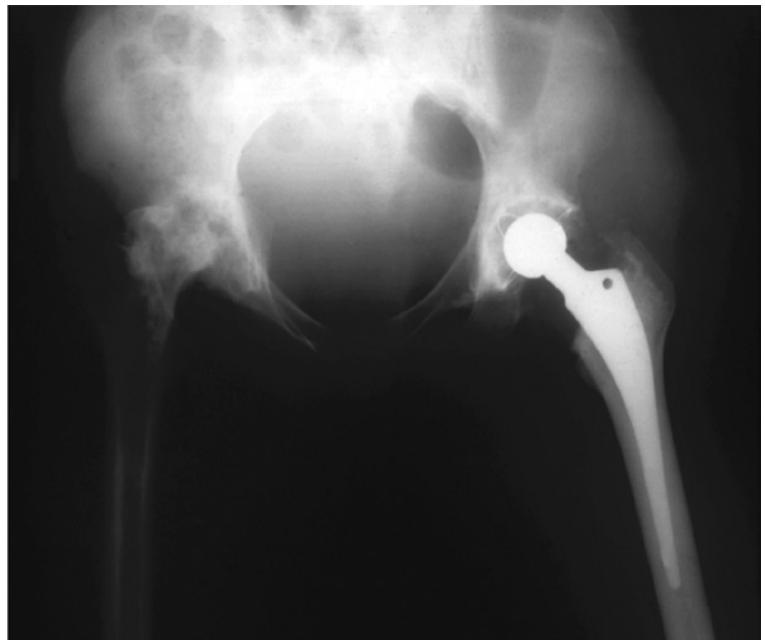


Fig. 4.36 Radiograph of hips and pelvis in a patient with sickle cell/haemoglobin C compound heterozygosity showing osteonecrosis of one hip resulting from vascular occlusion by sickle cells; the other hip has already been replaced because of the same process. (With thanks to Professor Irene Roberts.)

deafness and vestibular symptoms can occur [278, 284]. Altitude-related complications can occur, particularly splenic infarction [287]. Splenomegaly persists for longer so that splenic infarction and splenic sequestration can occur in adults as well as children while the onset of hyposplenism, resulting from recurrent splenic infarction, is delayed. In contrast to patients with sickle cell anaemia, in whom the spleen is atrophic as the result of infarction, patients with sickle cell/haemoglobin C disease occasionally suffer splenic infarction during aeroplane flights [288]. As a consequence of the delay in the development of hyposplenism, life-threatening infections are less common than in sickle cell anaemia. However, splenomegaly can lead to hypersplenism with chronic thrombocytopenia [289]. Priapism is common [285] but is less frequent than in sickle cell anaemia [290]. Painful crises, leg ulcers and renal impairment [278] are less common than in sickle cell anaemia. Growth retardation, which can be a feature of sickle cell anaemia, is not seen in sickle cell/haemoglobin C disease [291]. Because the red cell life span is considerably longer than in sickle cell anaemia, clinically apparent parvovirus-induced aplastic crises are uncommon and gallstones are less common. Fat embolism, which is more common than in sickle cell anaemia, can lead to worsening neurological, pulmonary and renal dysfunction, worsening anaemia, thrombocytopenia, an increased LDH and the presence of a leucoerythroblastic blood film with schistocytes; magnetic resonance imaging of the brain shows multiple hypodense punctate lesions [292].

Sickle cell/haemoglobin C disease is a hypercoagulable state, with an increased incidence of venous thromboembolism as well as arterial thrombosis and pregnancy-associated complications, including more frequent pre-eclampsia/eclampsia, need for induction of labour and small for gestational age babies [173, 293].

For unknown reasons, patients with sickle cell/haemoglobin C disease who contract

dengue fever may have a much more severe illness than control subjects or those with sickle cell anaemia [294].

Laboratory features

Blood count

The Hb is higher than in sickle cell anaemia, ranging from about 80 g/l up to the top of the normal range [120]. In a personally observed series of 29 patients the range was 89 to 156 g/l with a mean of 122 g/l. In one large Brazilian series the mean Hb was 109 g/l in women (102–120) and 127 (117–137) in men [284]. In another Brazilian series, the mean Hb (and interquartile range) in 361 children and adults with sickle cell/haemoglobin C disease was 110 (104–118) in comparison with 81 g/l (74–89) in 638 patients with sickle cell anaemia who were not taking hydroxycarbamide [290]. In a Canadian cohort of 104 adults, median Hb was 119 g/l [285]. A concentration of 100 g/l gives a fairly good separation between sickle cell anaemia and sickle cell/haemoglobin C disease. In one large study of adult patients originating in North Africa, West Africa and the Caribbean area, there were no individuals with sickle cell haemoglobin C disease with an Hb less than 110 g/l [295]. In children, splenomegaly has been associated with a lower Hb and platelet count [289]. The MCV is lower than in sickle cell anaemia with a mean level around the lower limit of the normal range [284, 296, 297]. The MCH is similar whereas the MCHC is more often elevated and the percentage of hyperdense cells is higher. On density gradient analysis, red cells of compound heterozygotes (SC) are denser than those in sickle cell anaemia (SS) and only slightly less dense than those in haemoglobin C disease (CC) [298]. The RDW is increased but generally less than in sickle cell anaemia [219, 299]. The HDW is increased [299]. The reticulocyte count is less markedly elevated than in sickle cell anaemia, with a mean level around 3–6%. The accuracy of measurement of red cell indices in sickle cell/haemoglobin C disease is dependent on

the automated instrument used; cells in this disease are less deformable than normal, leading to a false elevation of the MCV and reduction of the MCHC on impedance counters and on some earlier light-scattering instruments [299].

Splenic sequestration is associated with a fall not only in the Hb but also in the platelet count [289]. Bone marrow necrosis with fat embolism syndrome is associated with worsening anaemia and thrombocytopenia [283].

Individuals of African descent with sickle cell/haemoglobin C disease show a similar prevalence of α thalassaemia trait to those without this condition. The prevalence has varied between 20% and 35% in different series of patients [282]. Coexisting α thalassaemia trait leads to a higher RBC and a lower MCV and MCH in comparison with other patients with sickle cell/haemoglobin C disease. In contrast to sickle cell anaemia, concomitant α thalassaemia trait does not alter the Hb [114, 282, 295, 300], but a lower reticulocyte count and lower LDH indicate that there is less haemolysis [295]. Some clinical features may be ameliorated by the interaction [282, 301].

The WBC, neutrophil count and monocyte count are elevated in sickle cell/haemoglobin C disease but less so than in sickle cell anaemia [223].

When sickle cell/haemoglobin C disease is treated with hydroxycarbamide there is an

increase in the MCV and a fall in the MCHC and the proportion of hyperdense cells. The reticulocyte count falls. Regular venesection is sometimes used in patients with high haemoglobin levels, greater than 110 g/l, to induce iron deficiency and reduce the haematocrit, with a view to reducing whole blood viscosity and protecting against microvascular complications including retinopathy and hearing loss [302].

Blood film

The peripheral blood features of sickle cell/haemoglobin C disease are compared with those of sickle cell anaemia and haemoglobin C disease in Table 4.9 [236]. In contrast to sickle cell anaemia, the blood film less often shows classic sickle cells. Boat-shaped cells are more common than classic sickle cells but they also are less common than in sickle cell anaemia. Occasional cells may contain straight-edged six-sided haemoglobin C crystals. Around half of patients with sickle cell/haemoglobin C disease show characteristic poikilocytes (Fig. 4.37a,b), which are not seen in either sickle cell anaemia or haemoglobin C disease [223, 303]. These misshapen cells have complex forms. Some have crystals of varying shape and size jutting out at various angles. Others are curved, thus resembling

Table 4.9 The blood film features of sickle cell anaemia, sickle cell/haemoglobin C disease and haemoglobin C disease or $C\beta^0$ thalassaemia. ([237]/with permission of John Wiley & Sons.)

Genotype	SS	SC	CC or $C\beta^0$ thalassaemia
Number of cases	29	29	10
Sickle cells	24	6	0
Boat-shaped cells	24	16	1
Haemoglobin C crystals	0	4	5
SC poikilocytes	0	16	0
Irregularly contracted cells	5	25	9
Howell-Jolly bodies	29	5	0
Pappenheimer bodies	25	7	1
Target cells	27	29	9
Spherocytes	10	3	1
Polychromasia	24	9	3
NRBC	26	15	7

NRBC, nucleated red blood cells.

sickle cells, but also appear to contain crystals with straight edges or with blunt-angled rather than pointed ends. Haemoglobin C will copolymerise with haemoglobin S (as occurs in the rare sickle cells and in the more common boat-shaped cells in the compound heterozygous state) [304]. Haemoglobin S will cocrystallise with haemoglobin C (as occurs in the less common cells containing haemoglobin C crystals) [305]. Deoxygenation favours S-like polymerisation whereas oxygenation favours C-like crystallisation [304, 306]. It seems likely that the formation of SC poikilocytes is consequent on both processes occurring

simultaneously in the one cell. On scanning electron microscopy, forms seen include folded cells (compared to a taco), triconcave cells and stomatocytes [279].

Features of hyposplenism such as Howell-Jolly bodies and Pappenheimer bodies are less common than in sickle cell anaemia. Polychromasia and NRBC are likewise less common whereas target cells (Fig. 4.37c) show a similarly high frequency and irregularly contracted cells (Fig. 4.37d) are much more common. Hemighosts may be present (Fig. 4.37d). Assessment of the blood count and film usually permits the distinction between sickle cell/haemoglobin C

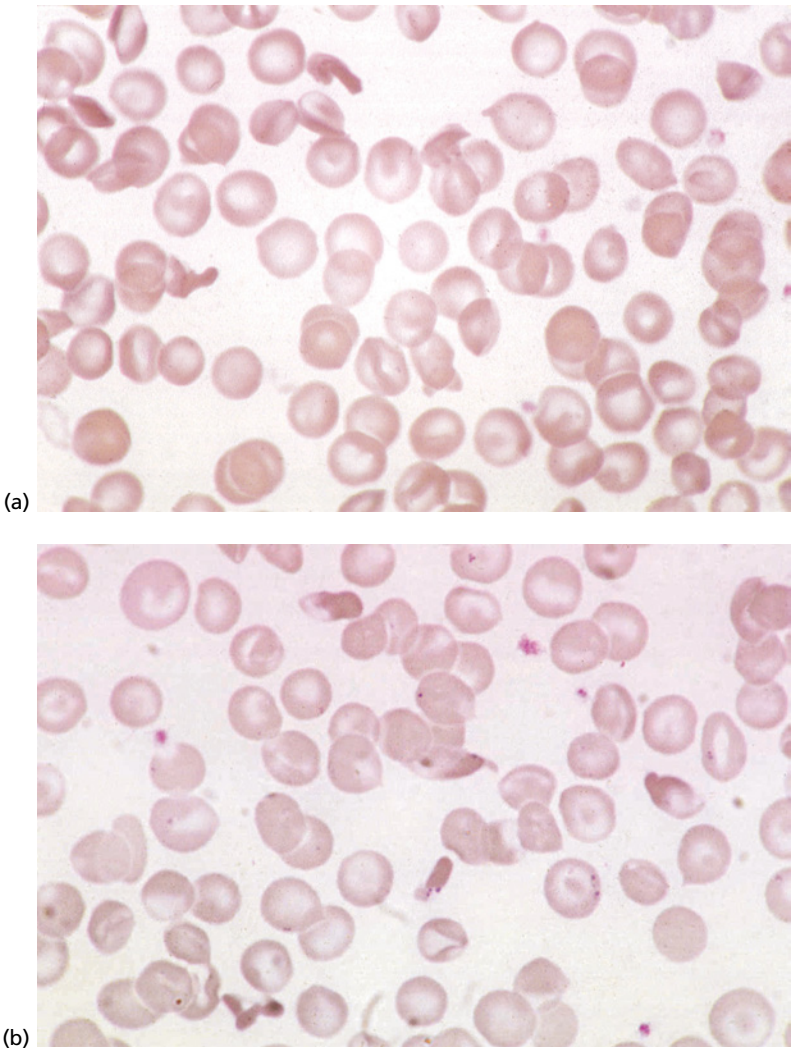
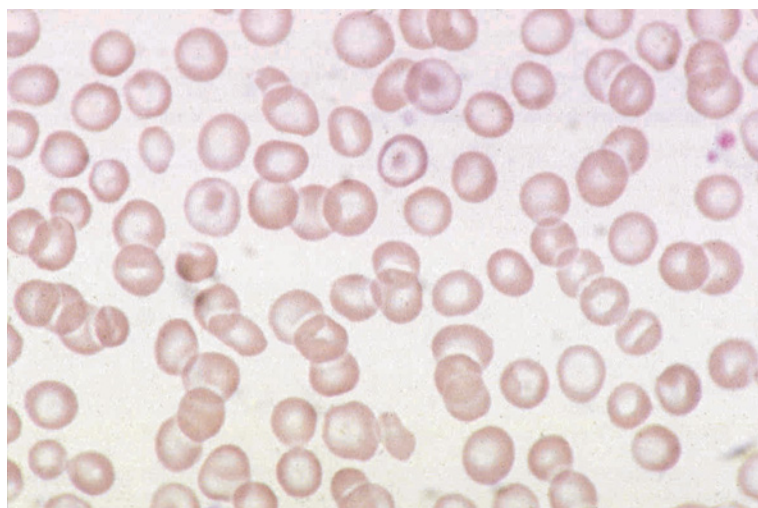
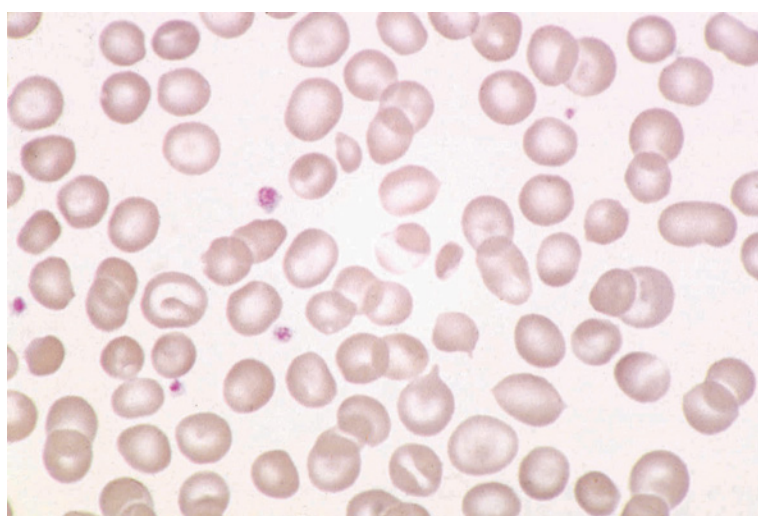


Fig. 4.37 Blood films from four patients with sickle cell/haemoglobin C compound heterozygosity showing the range of abnormalities that may be seen: (a) target cells and SC poikilocytes; (b) SC poikilocytes and a boat-shaped cell; (Continued on p. 257.)



(c)



(d)

Fig. 4.37 *Continued.*
 (c) hypochromia and target cells; (d) irregularly contracted cells, a hemi-ghost, a target cell and a stomatocyte. MGG $\times 100$.

disease and sickle cell anaemia. However, those cases that lack sickle cells, boat-shaped cells and SC poikilocytes (Fig. 4.37d) can be difficult to distinguish from haemoglobin C disease.

Bone marrow necrosis with a fat embolism syndrome is associated with a leucoerythroblastic blood film with small numbers of schistocytes [283].

Other investigations

Haemoglobin S and C are present in similar proportions (Figs 4.38–4.40). The haemoglobin F percentage ranges from normal to slightly elevated, with mean values of 1.1–3.3% having

been reported in different studies. The F percentage is significantly higher in females than in males [295]. As in sickle cell anaemia, the haemoglobin F percentage is affected by the β gene haplotype, averaging 3.2% with the Senegal haplotype and 1.5% and 1.4% respectively with the Benin and Bantu haplotypes [295]. In 98 UK subjects, the mean haemoglobin F was 1.46% (SD 1.81) with adult levels being reached by nine years of age [232]. The percentage of F cells is increased; in one study the mean level was 27% (range 5–73%), in comparison with normal levels of 0.5–7% [267]. Little information is available on the haemoglobin A₂ percentage in sickle cell/haemoglobin C disease since on cellulose

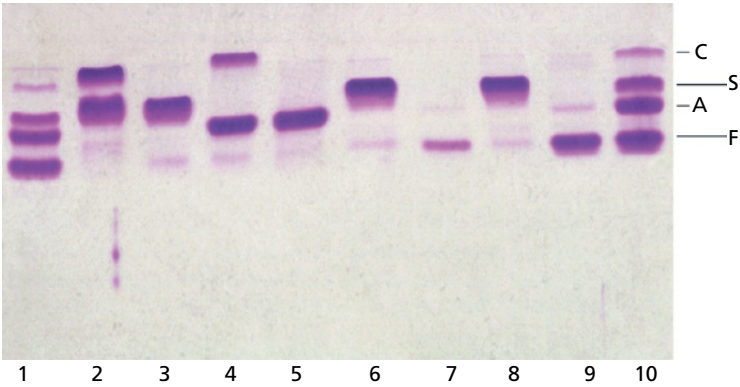


Fig. 4.38 Haemoglobin electrophoresis on agarose gel at pH6.2 showing a patient with sickle cell/haemoglobin C disease (second lane from left); lanes 1 and 10 show a control sample with, from below up, haemoglobins F, A, S and C.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	3.3*	---	1.13	60111
A ₀	---	1.0	2.39	18515
A ₂	4.6*	---	3.63	76445
S-window	---	46.3	4.52	879201
C-window	---	45.5	5.17	864100

Total Area: 1,898,372

F Concentration = 3.3* %

A₂ Concentration = 4.6* %

*Values outside of expected ranges

Analysis comments:

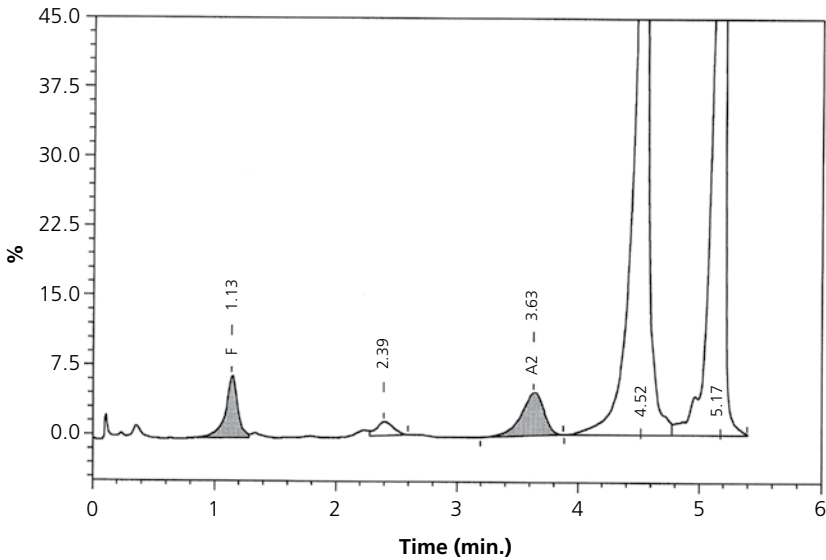


Fig. 4.39 HPLC chromatogram (Bio-Rad Variant II) in sickle cell/haemoglobin C disease, showing small early peaks representing acetylated haemoglobin F, haemoglobin F₀ (shaded), post-translationally modified haemoglobin S in the A₀ window, haemoglobin A₂ (shaded), haemoglobin S and haemoglobin C (with a shoulder representing post-translationally modified haemoglobin C).

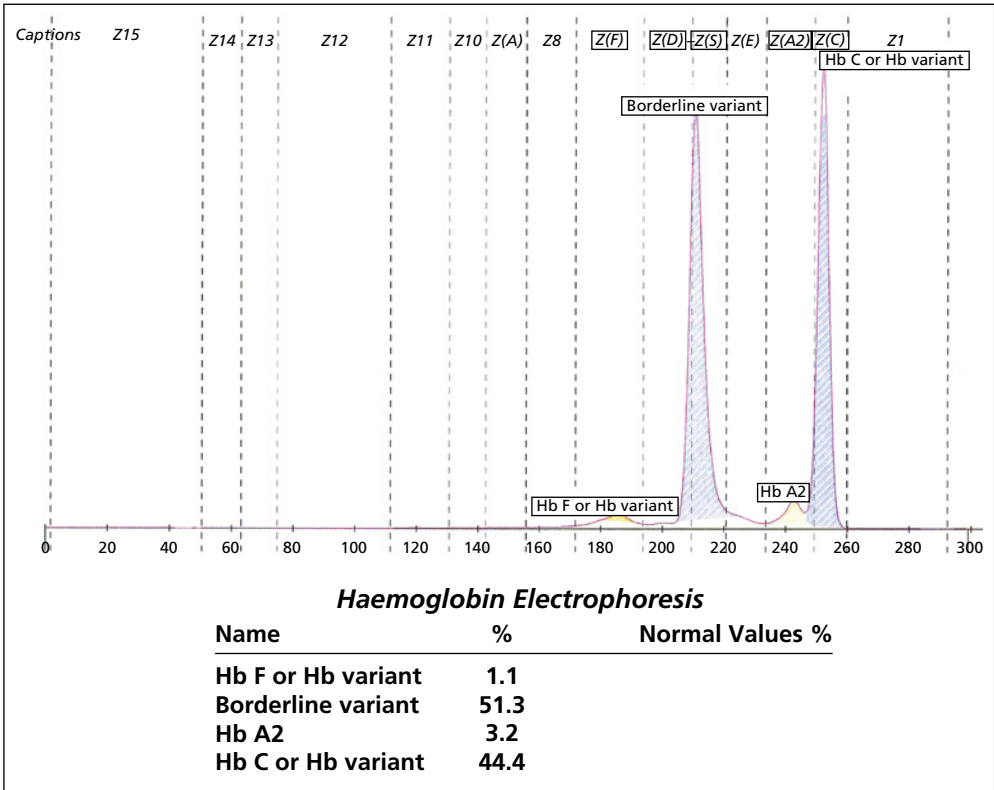


Fig. 4.40 Capillary electrophoresis in sickle cell/haemoglobin C disease, showing haemoglobins F, S, A₂ and C. Note that the A₂ and C peaks overlap.

acetate electrophoresis haemoglobin A₂ comigrates with haemoglobin C and on HPLC some post-translationally modified haemoglobin S falls into the A₂ window.

The sickle solubility test is positive and immunoassays demonstrate the presence of haemoglobins S and C with no haemoglobin A.

Bilirubin is normal or mildly elevated. LDH is elevated in comparison with control subjects but is less elevated than in sickle cell anaemia. Red cell life span ranges from moderately shortened to slightly less than normal. The oxygen dissociation curve shows reduced oxygen affinity (i.e. a right shifted curve) and a higher P₅₀. The reduction in oxygen affinity is less than is seen in sickle cell anaemia [254].

As for sickle cell anaemia, vaso-occlusive episodes can be complicated by haemophagocytic lymphohistiocytosis and the bone marrow then shows haemophagocytosis [257].

Diagnosis

Diagnosis rests on demonstrating the presence of haemoglobin S and haemoglobin C with haemoglobin A being absent. The identity of the two variant haemoglobins must be confirmed by at least two independent techniques. When electrophoresis is the primary technique, it is important not to confuse compound heterozygous states for S and C-Harlem, O-Arab or E (see pages 270, 268 and 273) with sickle cell/haemoglobin C disease since all have two bands in the same positions on cellulose acetate at alkaline pH. In compound heterozygosity for haemoglobins S and E, the band in the C position constitutes a lower percentage than the S band. Homozygous S with coexisting G-Philadelphia will also have bands in the positions of S and C but the band in the C position, which represents the hybrid

$\alpha^{\text{G-Philadelphia}}\beta^{\text{S}}$ haemoglobin, constitutes an appreciably lower percentage than the band representing S plus G-Philadelphia.

Interactions with other haemoglobinopathies and other haematological diseases

There is conflicting evidence as to the effect of coexisting α thalassaemia trait. In most series of patients α thalassaemia trait has been associated with a lower risk of osteonecrosis, retinopathy, gallbladder disease and painful crises [282, 293]. The risk of splenic sequestration is greatly reduced [307].

Individuals with sickle cell/haemoglobin C disease who are also heterozygous for the α chain variant haemoglobin G-Philadelphia have disease of variable severity. One reported case was more severe than is usual in sickle cell/haemoglobin C disease [308] while another had a mild clinical course with abundant crystals in circulating cells and numerous folded cells [309]. The latter is considered the more typical clinical picture, attributable to the presence of the G-Philadelphia α chain both increasing the likelihood of crystallisation of haemoglobin C and decreasing the likelihood of polymerisation of haemoglobin S [279]. Haemoglobin electrophoresis is complex. At alkaline pH there are bands with the mobility of S (about 35%), C (about 47%) and a slow G/C hybrid (about 15%) [308]. The 'S' band represents S and G-Philadelphia. The 'C' band represents C and S/G hybrid. At acid pH there are two bands with the mobility of S and C. A similar complexity is seen on HPLC.

A severe phenotype has been observed with coincidental hereditary spherocytosis [243].

Sickle cell/ β thalassaemia

Sickle cell/ β thalassaemia is a compound heterozygous state for β^{S} and either $\beta^{\text{+}}$ thalassaemia or β^0 thalassaemia [310, 311]. A rare cause of an S/ β^0 genotype is coinheritance of β^{S} with deletion of the locus control region β in *trans* [85]. In sickle cell/ β^0 thalassaemia there is no haemoglobin A whereas in sickle cell/ $\beta^{\text{+}}$

thalassaemia a variable amount of haemoglobin A is present, depending on the severity of the $\beta^{\text{+}}$ thalassaemia variant. The reduced concentration of haemoglobin S within the red cell, together with the greater or lesser increase in percentages of haemoglobins A₂ and F, lessens the likelihood of sickling and lessens the haemolysis (in comparison with sickle cell anaemia) but this is counterbalanced by the higher Hb and increased blood viscosity.

Clinical features

Patients with sickle cell/ β^0 thalassaemia have less evidence of haemolysis than patients with sickle cell anaemia but despite this the frequency of painful crises is, if anything, greater [200]. The explanation may lie in the higher haemoglobin concentration. However, the incidences of acute chest syndrome and cerebrovascular disease have been found to be significantly lower in children with the compound heterozygous state [312]. Patients with sickle cell/ $\beta^{\text{+}}$ thalassaemia may have both less haemolysis and a reduced incidence of painful episodes in comparison with sickle cell anaemia. These patients have a very variable phenotype depending on the nature of the β thalassaemia allele, and the disease tends to be less severe as the percentage of haemoglobin A increases. At one end of the spectrum, severe $\beta^{\text{+}}$ thalassaemia variants, such as the common IVS1-5(G>C) mutation, make very little haemoglobin A and produce a phenotype very similar to sickle cell/ β^0 thalassaemia; contrastingly, mild forms of $\beta^{\text{+}}$ thalassaemia, such as the -29(A>G) mutation, result in only a small reduction in the synthesis of β^{A} globin, and cause a very mild form of sickle cell disease, associated with few acute complications and a reduced risk of life- or organ-threatening complications. Patients may, however, have a higher incidence of proliferative retinopathy, as a result of the higher Hb [243]. Sickle cell/ β thalassaemia is generally more severe in Mediterranean populations than in those of African descent because of the greater prevalence of mild $\beta^{\text{+}}$ thalassaemia variants in the latter group. This type of mild sickle cell/ $\beta^{\text{+}}$

thalassaemia is sometimes designated sickle cell/ β^{++} thalassaemia. Splenomegaly persists longer than in sickle cell anaemia, particularly in those with sickle cell/ β^+ thalassaemia, and surgical splenectomy is performed in up to 30% of patients. Patients with sickle cell/ β^+ thalassaemia and persisting splenomegaly remain susceptible to splenic infarction during aeroplane flights whereas those with sickle cell/ β^0 thalassaemia resemble patients with sickle cell anaemia since they are likely to have suffered recurrent splenic infarction and consequent atrophy and therefore do not remain susceptible [288]. Sometimes massive splenomegaly leads to hypersplenism. Overexpansion of the bone marrow cavity in the skull can cause frontal bossing. In one study children with sickle cell/ β^0 thalassaemia had a lower transcranial Doppler velocity than those with sickle cell anaemia, suggesting less vasculopathy although the incidence of silent cerebral infarction did not differ [312]. Transfusion-transmitted babesiosis can cause severe haemolysis in S/ β^0 thalassaemia, as well as in sickle cell anaemia [172]. S/ β^0 thalassaemia has a similar survival to sickle cell anaemia (estimated at 58 years in one US study [195]) while S/ β^+ thalassaemia has a longer survival, similar to that of compound heterozygosity for S and C (estimated at 66 years) [195]. However, estimates depend on the precise statistical techniques used [139].

Laboratory features

Blood count

Anaemia is milder than in sickle cell anaemia, the Hb varying from about 50 g/l to within the normal range, depending on the coinherited β thalassaemia variant. The distribution of Hb is bimodal, being higher in those with sickle cell/ β^+ thalassaemia than in those with sickle cell/ β^0 thalassaemia; mean values in one study were 107 and 81 g/l respectively [13]. The mean Hb is significantly higher in sickle cell/ β^0 thalassaemia than in sickle cell anaemia, with mean levels of 92 and 81 g/l respectively in one study [312]. The MCV, MCH and MCHC are

reduced, again showing a bimodal distribution. Mean values observed for sickle cell/ β^+ thalassaemia and sickle cell/ β^0 thalassaemia respectively were 72 and 69.8 fl for MCV, 22.6 and 20.1 pg for MCH and 315 and 288 g/l for MCHC [311]. For both groups mean values for MCV, MCH and MCHC are lower than mean values in sickle cell anaemia but overlap occurs. The RDW is markedly increased in sickle cell/ β^0 thalassaemia and moderately increased in sickle cell/ β^+ thalassaemia [219]. It should be noted that in patients with sickle cell/ β thalassaemia who develop megaloblastic anaemia, the MCV and MCH, although elevated in comparison with baseline values, may be within the normal range.

The reticulocyte count is elevated, in sickle cell/ β^0 thalassaemia to around 8–9% on average and in sickle cell/ β^+ thalassaemia to around 3% on average [296]. During complicating bacterial or parvovirus infection or megaloblastic anaemia the usual elevation of the reticulocyte count is lacking.

Coexisting α thalassaemia increases the Hb, MCV and MCH and reduces the reticulocyte count [13]. The RBC and haemoglobin A_2 percentage are lowered [313]. This alteration in the red cell indices and haemoglobin A_2 percentage not infrequently leads to misdiagnosis of S/ β^0 thalassaemia as sickle cell anaemia, particularly but not only in those with deletion of two α genes [313].

The monocyte count is on average higher than in control subjects [212].

Blood film

The blood film abnormalities are more severe in sickle cell/ β^0 thalassaemia (Fig. 4.41) than in sickle cell/ β^+ thalassaemia (Fig. 4.42). Classic sickle cells are quite uncommon, particularly in sickle cell/ β^+ thalassaemia. There are some boat-shaped cells. There is hypochromia and microcytosis and circulating NRBC show defective haemoglobinisation. Target cells are prominent and basophilic stippling may be apparent. Features of hyposplenism may be present, particularly in sickle cell/ β^0 thalassaemia. In patients with hyposplenism, Pappenheimer

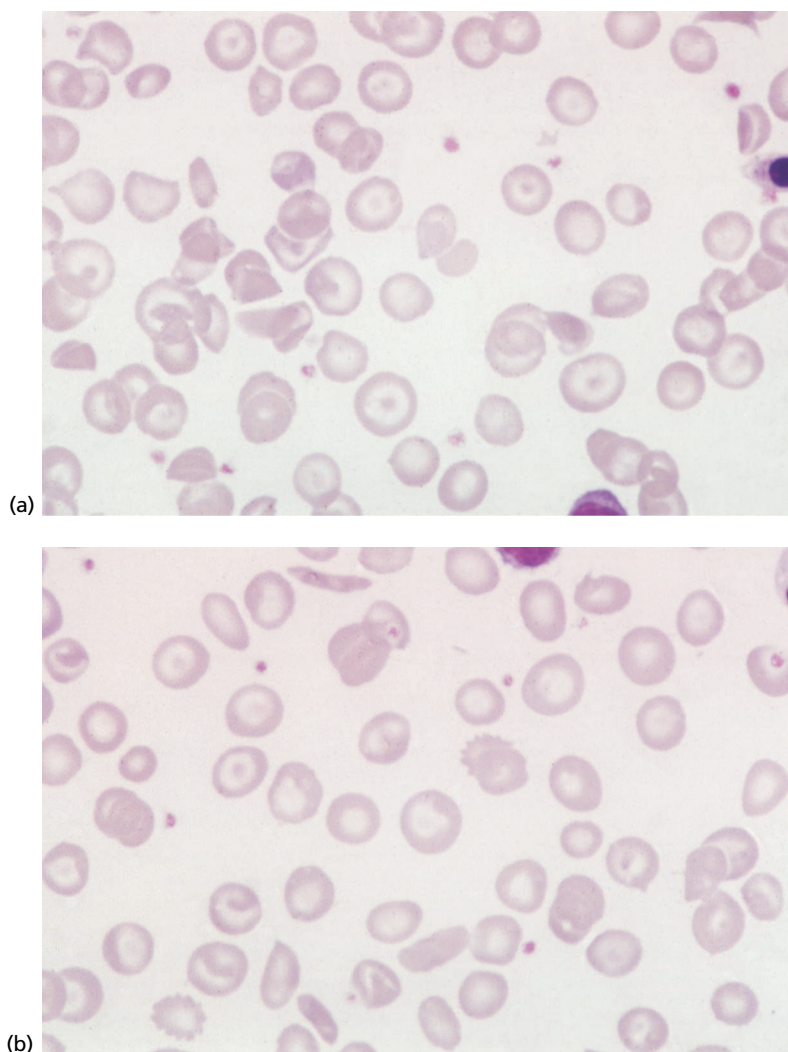


Fig. 4.41 Blood films in two patients with sickle cell/ β^0 thalassaemia showing: (a) target cells, a nucleated red blood cell and a number of partly sickled cells; (b) hypochromia, microcytosis, target cells and a number of partly sickled cells. MGG $\times 100$.

bodies are often very prominent. Polychromasia is present unless there is associated erythropoietic failure caused by infection or megaloblastosis.

Other tests

Haemoglobin S comprises more than 50% of total haemoglobin, in contrast to sickle cell trait when it is less than 50%. In patients with sickle cell/ β^0 thalassaemia there is no haemoglobin A whereas in those with sickle cell/ β^+ thalassaemia

(Figs 4.43–4.45) the amount of haemoglobin A varies from almost undetectable to, rarely, as high as 45% (Table 4.10) [310, 311, 314–317]. Haemoglobin F is usually 5–15% and the percentage of F cells is considerably increased. When the β^s gene is associated with the Arab-Indian haplotype, the mean haemoglobin F is about 18% with some values approaching 40% [318]. Compound heterozygosity for β^s and the African type of ($^A\gamma\delta\beta$) 0 thalassaemia has haemoglobin F of 22–34% [319]. As for sickle

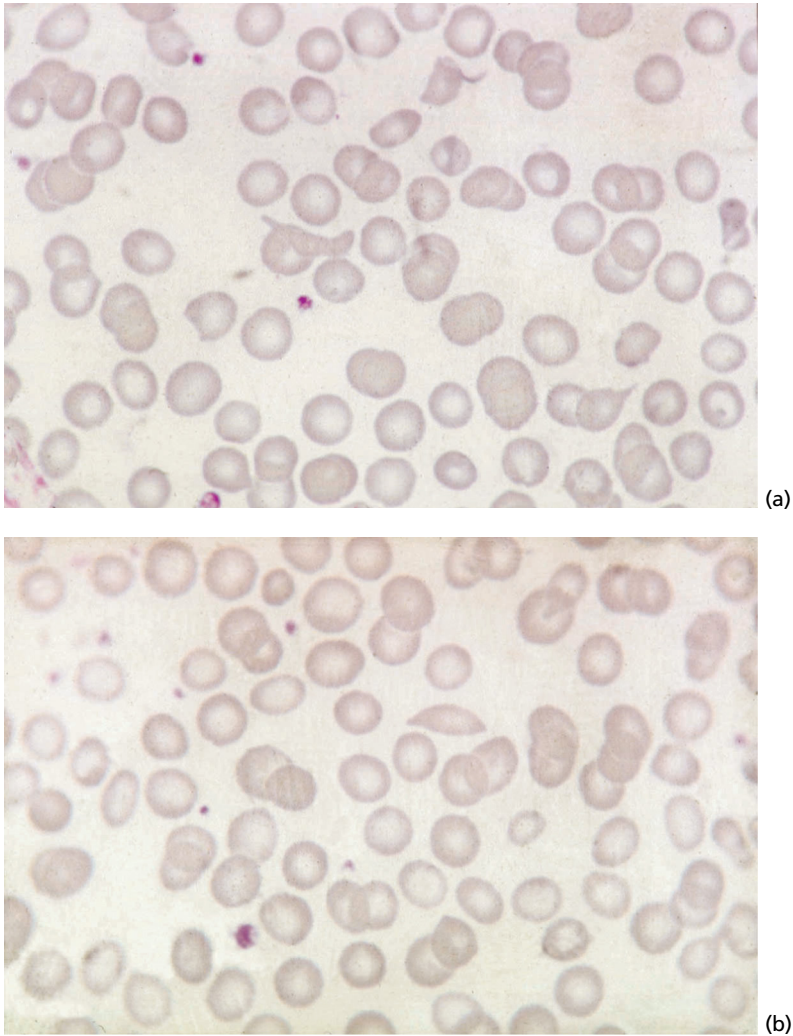


Fig. 4.42 Blood films in two patients with sickle cell/ β^+ thalassaemia showing: (a) hypochromia, poikilocytosis and a probable sickle cell; (b) hypochromia, microcytosis and one partly sickled cell. The first patient had 70% haemoglobin S, 24% haemoglobin A and 6% haemoglobin A₂; red cell indices were RBC $5.07 \times 10^{12}/l$, Hb 106 g/l, MCV 64 fl, MCH 21 pg and MCHC 329 g/l. The second patient had 59% haemoglobin S, 25% haemoglobin A plus A₂ and 13% haemoglobin F; the red cell indices were RBC $4.71 \times 10^{12}/l$, Hb 106 g/l, Hct 0.32, MCV 68 fl, MCH 22.5 pg and MCHC 330 g/l. MGG $\times 100$.

cell anaemia, the haemoglobin F concentration is associated with the β globin haplotype, being higher with the Senegal and Arab-Indian haplotypes. Because of the overlap in values, the haemoglobin F percentage is not useful in separating S/ β^0 thalassaemia from sickle cell anaemia; in one series of Jamaican patients the percentage F tended to be higher in the compound heterozygotes but the difference

was not significant [311]. Haemoglobin A₂ tends to be somewhat elevated, usually 3.5–5.5% with the level being higher when the β thalassaemia gene is a β^0 rather than a β^+ [320]. Higher levels of haemoglobins F and A₂ (and a milder clinical course) have been observed when the β thalassaemia mutation is a large (290 bp) deletion [321]. The higher level of haemoglobin A₂ in sickle cell/ β^0 thalassaemia can be useful in

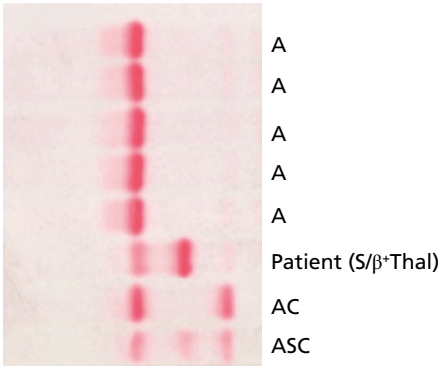


Fig. 4.43 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in a patient with sickle cell/ β^+ thalassaemia compound heterozygosity; ASC indicates a control sample containing haemoglobins A, S and C.

helping to make a distinction between the compound heterozygous state and sickle cell anaemia, with microcytosis consequent on coexisting α thalassaemia trait, in which haemoglobin A₂ is usually in the range of 2–4%. Although there is some overlap in haemoglobin A₂ percentages this is the most useful variable for making the distinction; the Hb, reticulocyte count and haemoglobin F percentage show more overlap (see Table 4.8). Where necessary, DNA analysis can be used to diagnose haemoglobin S/ β thalassaemia and determine the nature of the β thalassaemia variant.

Red cell life span is reduced, particularly in sickle cell/ β^0 thalassaemia, but not to the same extent as in sickle cell anaemia. The $\alpha\beta$ chain

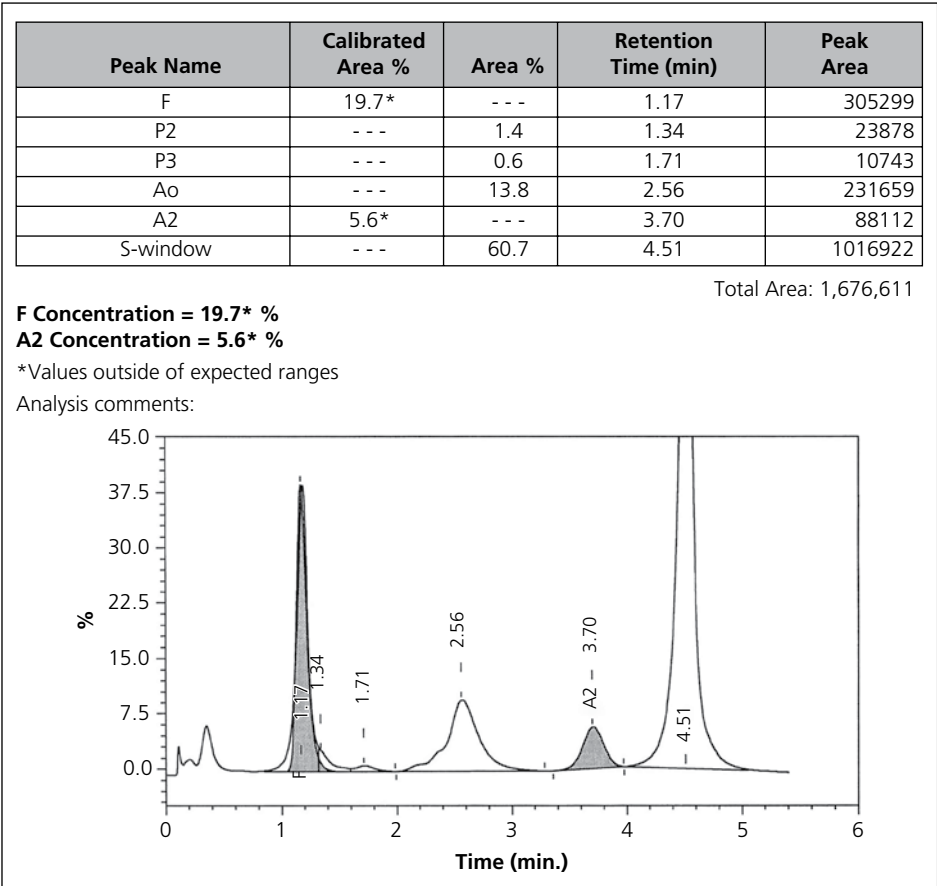


Fig. 4.44 HPLC chromatogram (Bio-Rad variant II) of haemoglobin S/ β^+ thalassaemia, showing a triple peak of post-translationally modified haemoglobin F, haemoglobin F₀ (shaded), low peaks of modified haemoglobin A, haemoglobin A_v, haemoglobin A₂ (shaded) and haemoglobin S. Note the shoulder on the left of the haemoglobin A₀ peak, which represents glycated haemoglobin S.

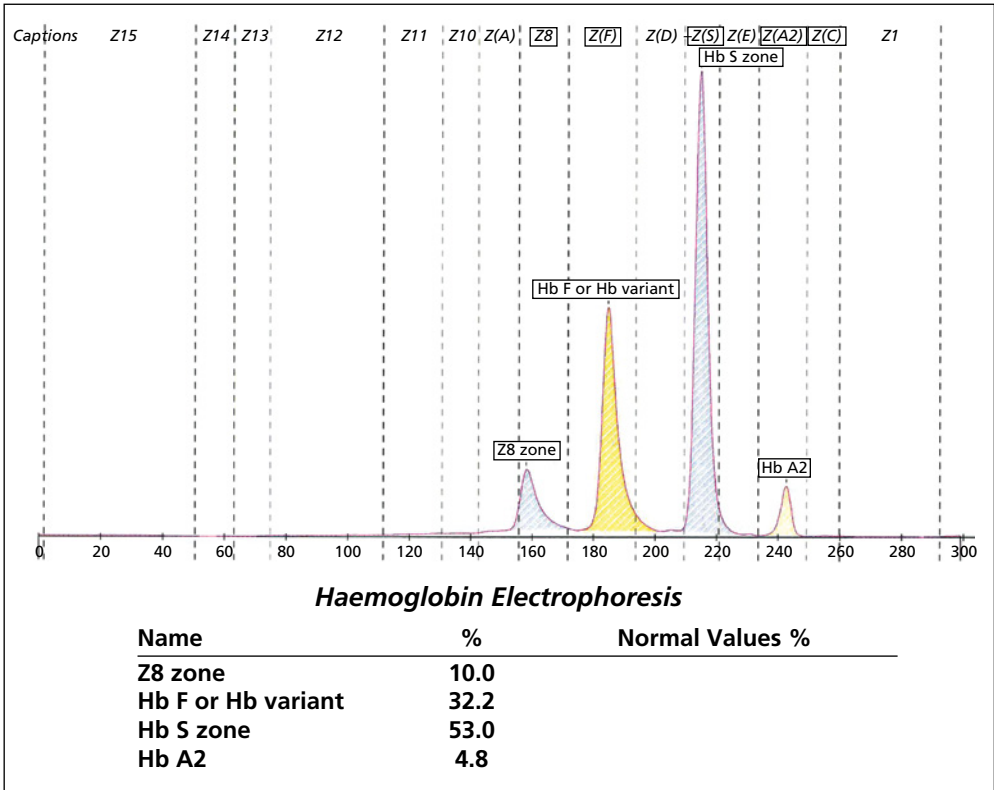


Fig. 4.45 Capillary electrophoresis (Sebia Capillaries 3) in sickle cell/ β^+ thalassaemia showing haemoglobins A (10%), F (32%), S (53%) and A_2 (4.8%).

Table 4.10 Percentage of haemoglobin A in compound heterozygosity for S and β^+ thalassaemia [310, 311, 314–317].

Mutation and ethnic group	Percentage of haemoglobin A
Severe (β^+)	
C \rightarrow G at IVS-II, position 745 (Greek/Turkish)	3–5
G \rightarrow C at IVS-1, position 5 (Indian)	3–5
Moderate (β^+)	
G \rightarrow A at IVS-1, position 110 (Greek/Turkish)	8–14
Mild (β^{++})	
C \rightarrow T at –88 (Black)	18–25
A \rightarrow G at –29 (Black)	18–25
G \rightarrow T at IVS-2, position 5 (Greek/Turkish)	18–25

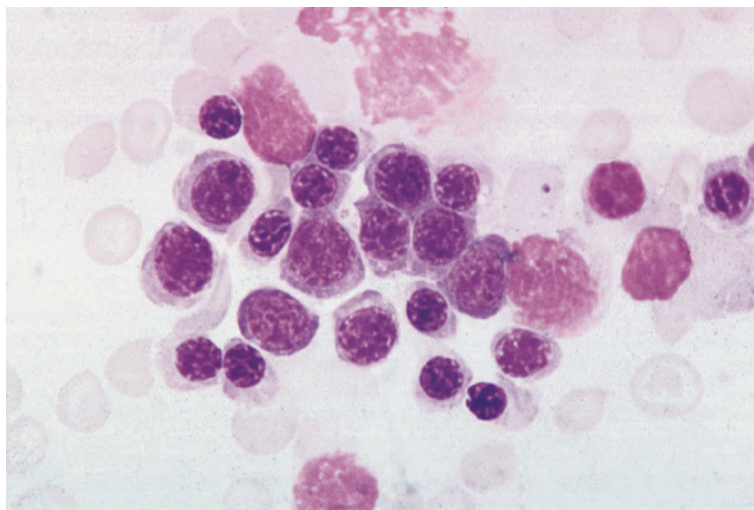


Fig. 4.46 Bone marrow aspirate in sickle cell/ β^0 thalassaemia compound heterozygosity showing erythroid hyperplasia and one sickle cell. MGG $\times 100$.

synthesis ratio in peripheral blood reticulocytes is increased in sickle cell/ β thalassaemia whereas it is normal in sickle cell anaemia.

In the neonatal period the diagnosis of sickle cell/ β^+ thalassaemia can be difficult [253]. Confusion with sickle cell trait can occur if almost all the haemoglobin present is haemoglobin F and the proportions of haemoglobins S and A are so low that it is not clear which is present in the greater amount. Neonates with sickle cell/ β^+ thalassaemia may also have only haemoglobins S and F detected so that confusion with sickle cell anaemia and sickle cell/ β^0 thalassaemia is possible. DNA analysis is needed to make a diagnosis in these circumstances; alternatively, if this is not available, family studies and follow-up are indicated.

The bone marrow aspirate (Fig. 4.46) shows erythroid hyperplasia, sickle cells and a variable degree of iron overload.

Diagnosis

Diagnosis of compound heterozygosity for haemoglobin S and β^+ thalassaemia is straightforward, merely requiring the demonstration of both haemoglobin A and haemoglobin S by two independent techniques and the demonstration that haemoglobin S is present as a larger proportion than haemoglobin A. Diagnosis of compound heterozygosity for

haemoglobin S and β^0 thalassaemia is more difficult since a distinction has to be made from sickle cell anaemia with microcytosis (e.g. due to coexisting α thalassaemia) (see earlier). The coexistence of α thalassaemia can lead to misdiagnosis of S/ β^0 thalassaemia as sickle cell anaemia [313]. A distinction also needs to be made from compound heterozygosity for haemoglobin S and deletional HPFH, particularly with coexisting α thalassaemia trait; in this instance clinical features, Hb, MCV and haemoglobin F percentage are useful (see Table 4.8). When a precise diagnosis is important, e.g. for genetic counselling, and the diagnosis is not clear from family studies and from a consideration of the proportions of various haemoglobins, DNA analysis should be carried out.

Other causes of sickle cell disease

Sickle cell/haemoglobin D-Punjab/Los Angeles disease

Compound heterozygosity for sickle cell haemoglobin and haemoglobin D-Punjab (D-Los Angeles) leads to sickle cell disease which is, on average, slightly milder than sickle cell anaemia [13, 273, 322–326]. Some patients are asymptomatic [327]. This compound heterozygous state has been observed in African Americans, African Caribbeans, Central and

South Americans (Mexicans and Venezuelans), Arabs and Turks and, in addition, in a number of individuals of mixed ancestry (northern European/American Indian, English/African, English/African Caribbean), including several individuals who appeared to have only Mediterranean or northern European ancestry. The clinical features are of a mild or moderate haemolytic anaemia with acute vaso-occlusive episodes. Persisting splenomegaly is more common than in sickle cell anaemia. The Hb is usually between 50 and 100 g/l and the reticulocyte count between 5% and 20%

(occasionally higher). The MCV is very variable but macrocytosis is quite common, with some individuals having an MCV of 110–120 fl. The blood film (Fig. 4.47) shows anisocytosis, poikilocytosis, target cells, sickle cells, boat-shaped cells, nucleated red cells and sometimes macrocytes. The bone marrow shows erythroid hyperplasia and sickle cells (Fig. 4.48). On cellulose acetate electrophoresis at alkaline pH, haemoglobins S and D-Punjab show the same electrophoretic mobility but HPLC (Fig. 4.49) and electrophoresis at acid pH separate these two haemoglobins from each other. On capillary

Fig. 4.47 Blood film in sickle cell/haemoglobin D-Punjab compound heterozygosity. MGG $\times 100$.

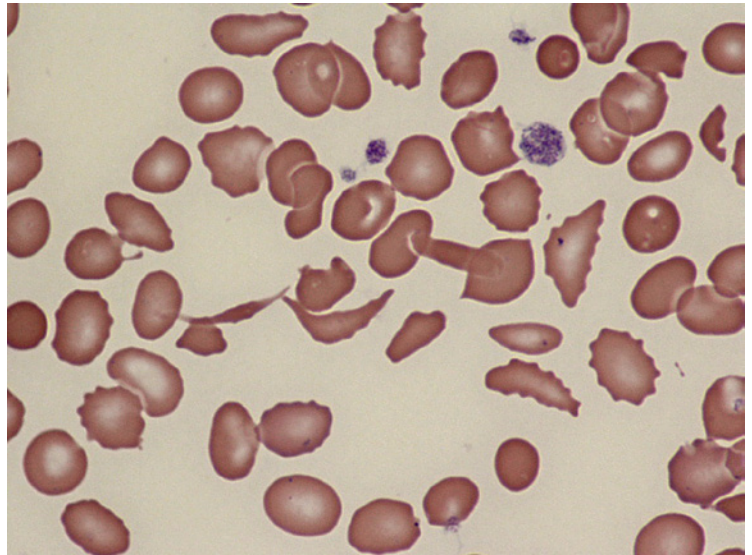
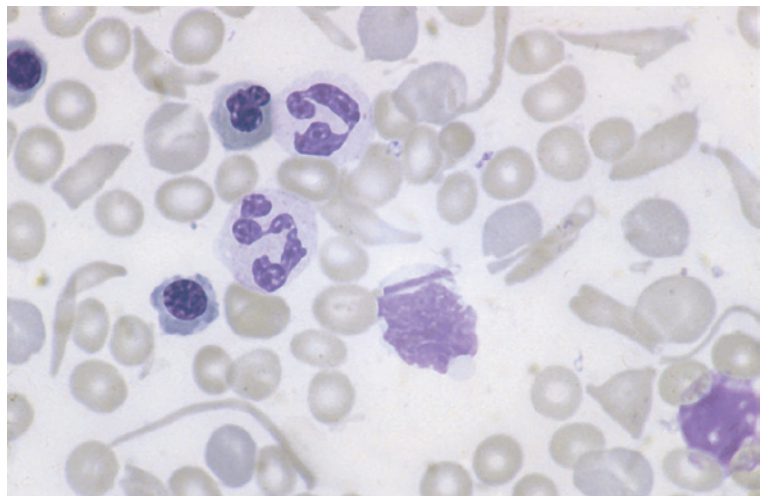


Fig. 4.48 Bone marrow aspirate in sickle cell/haemoglobin D-Punjab compound heterozygosity showing prominent sickle cell formation. MGG $\times 100$.



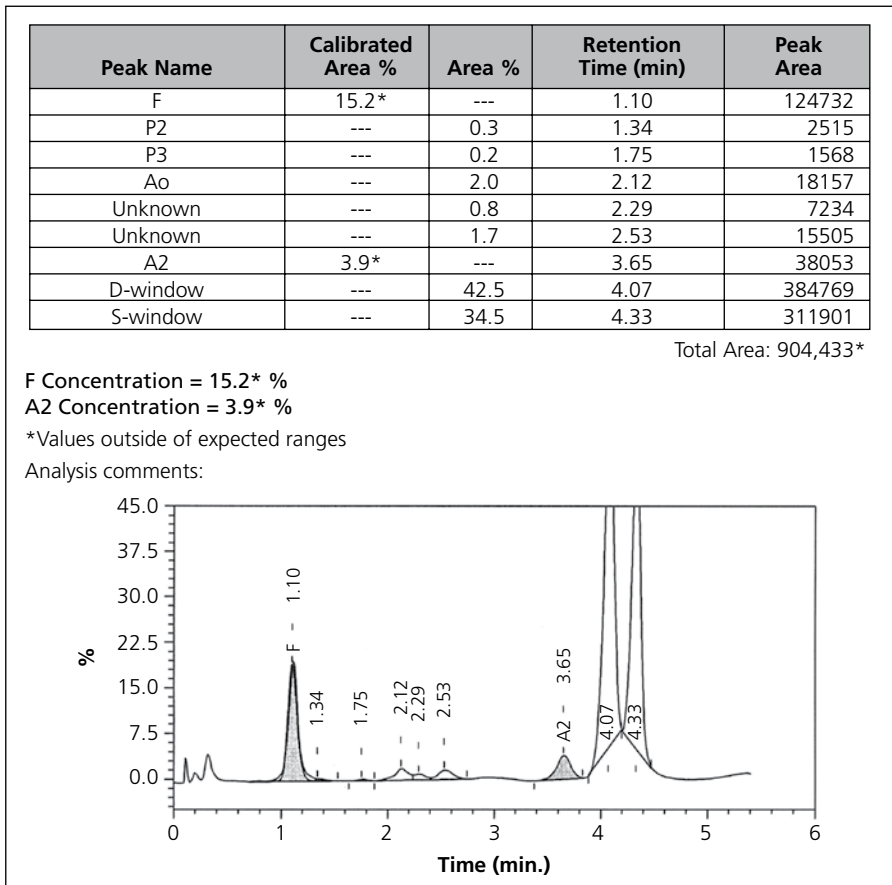


Fig. 4.49 HPLC chromatogram (Bio-Rad variant II) in sickle cell/haemoglobin D-Punjab compound heterozygosity. The peaks, from left to right, are post-translationally modified haemoglobin F (three peaks), haemoglobin F₀ (shaded), post-translationally modified haemoglobins S and D-Punjab (three peaks), haemoglobin A₂ (shaded), haemoglobin D and haemoglobin S.

electrophoresis the S and D-Punjab peaks overlap (Fig. 4.50). Haemoglobin D forms a somewhat higher proportion of total haemoglobin than does haemoglobin S [326]. In a few cases haemoglobin F has been significantly elevated (e.g. 13–20%) [325], but usually haemoglobin F is present in only small amounts. Haemoglobin A₂ may be slightly elevated [326]. As for all types of sickle cell disease, this compound heterozygous state is very variable, with one study from Kuwait suggesting that it may not be ameliorated by high haemoglobin F levels [328].

It should be noted that coinheritance of haemoglobin S and haemoglobin D variants other than haemoglobin D-Punjab does not cause

sickle cell disease. For example, two Nigerians with haemoglobin S/haemoglobin D-Ibadan were asymptomatic [324]. Similarly, haemoglobin D-Iran does not interact adversely with haemoglobin S. DNA analysis is typically used to distinguish haemoglobin D-Punjab from other D haemoglobins, this being particularly important in the context of antenatal screening.

Median survival is similar to that of sickle cell anaemia.

Sickle cell/haemoglobin O-Arab disease

Compound heterozygosity for sickle cell haemoglobin and haemoglobin O-Arab ($\alpha_2\beta_2$ 2^{121Glu→Lys}) leads to sickle cell disease that is

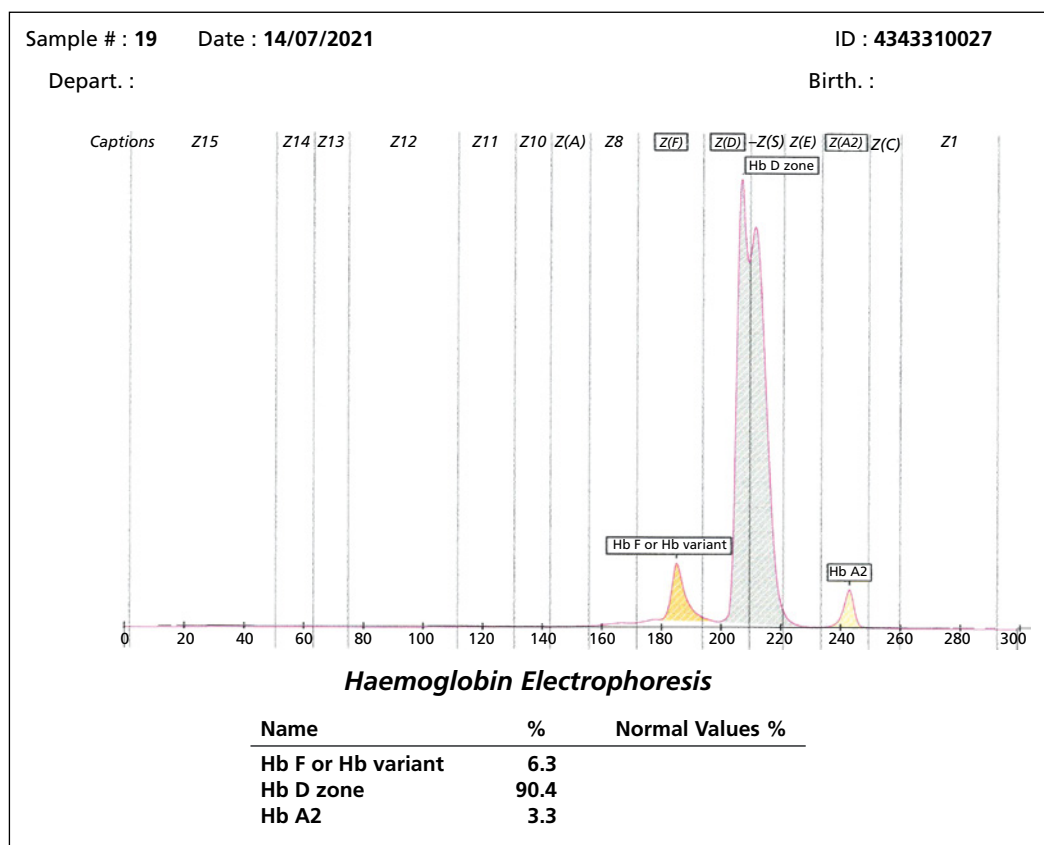


Fig. 4.50 Capillary electrophoresis (Sebia Capillars) in sickle cell/haemoglobin D-Punjab compound heterozygosity showing overlapping peaks of S and D-Punjab.

generally severe [13, 273, 329–331]. Sickle cell/haemoglobin O-Arab has been observed in Arabs, Africans (Sudanese and Kenyans), African Caribbeans, African Americans and Americans who appeared to be of Caucasian ancestry. The Hb in adults varies between 61 and 99 g/l. The reticulocyte count is usually between 8% and 10% (1–15% reported). Reported MCVs have been quite variable, from normal to moderately macrocytic levels (82–110 fl in adults). The blood film (Fig. 4.51) is similar to that in sickle cell anaemia. Oxygen affinity is reduced, comparable to what is seen in sickle cell anaemia. On electrophoresis on cellulose acetate at alkaline pH, haemoglobin O-Arab has similar mobility to haemoglobin C (Fig. 4.52) but at acid pH mobility depends on the electrophoresis medium. On agarose gel it

is slightly slower than S (Fig. 4.53). On HPLC there are two abnormal peaks, one in the position of S and the other between S and C (Fig. 4.54). Capillary electrophoresis also shows two distinct peaks (Fig. 4.55). Haemoglobin O-Arab and haemoglobin C-Harlem can be easily confused with each other when present in the compound heterozygous state with haemoglobin S. The difference in mobility on citrate agar at acid pH is useful in making the distinction (Table 4.11). Retention times on HPLC are quite similar, 4.9–4.93 minutes for O-Arab and 4.89 minutes reported for C-Harlem on a Bio-Rad Variant II instrument [332]. Haemoglobin S forms a somewhat higher proportion of total haemoglobin than does haemoglobin O-Arab [326]. DNA analysis can be used for definitive diagnosis.

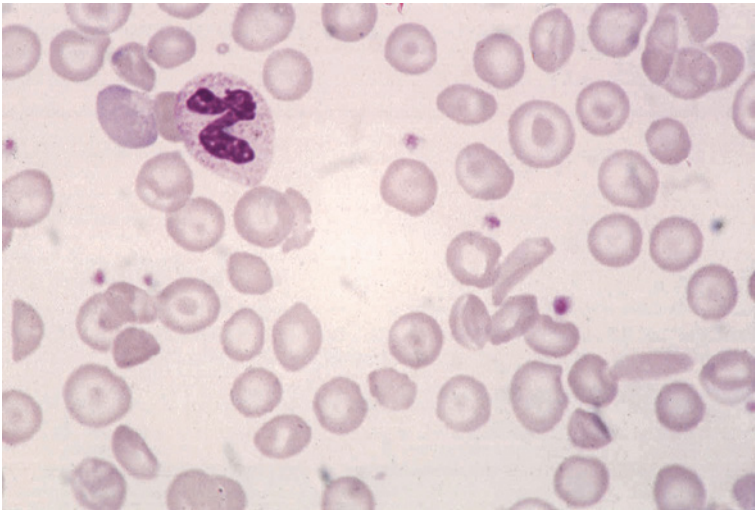


Fig. 4.51 Blood film in sickle cell/haemoglobin O-Arab compound heterozygosity showing hypochromia, target cells and partially sickled cells. (Note: O-Arab in this patient was misidentified as C-Harlem in the first edition of this book; the correct identity was subsequently confirmed by family studies, citrate agar electrophoresis and mass spectrometry.) MGG $\times 100$.

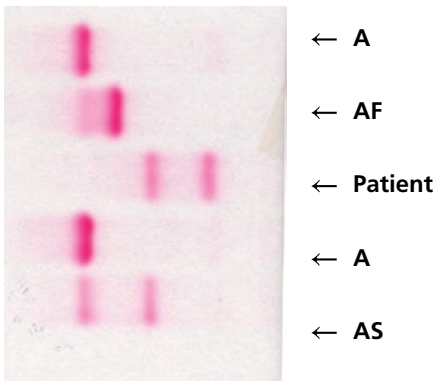


Fig. 4.52 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in sickle cell/haemoglobin O-Arab compound heterozygosity; by this technique the pattern cannot be distinguished from that of sickle cell/haemoglobin C compound heterozygosity.

Sickle cell/haemoglobin C-Harlem compound heterozygosity

This condition is slightly milder than sickle cell anaemia. The blood film shows similar features. Haemoglobin electrophoresis at alkaline pH resembles that of sickle cell/haemoglobin C disease whereas at acid pH on citrate agar (but not agarose gel) there is a single band with the mobility of haemoglobin S (see Table 4.11).

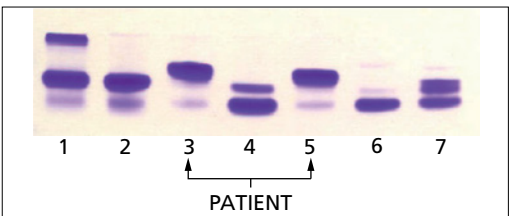


Fig. 4.53 Haemoglobin electrophoresis on agarose gel at acid pH in sickle cell/haemoglobin O-Arab compound heterozygosity (lanes 3 and 5) showing a faint F band and broadening of the S band in the direction of C by the presence of O-Arab. From left to right, lanes are: (1) F, A and C; (2) F and A; (3) S plus O-Arab; (4) F and A; (5) S plus O-Arab; (6) F; (7) A and S. The mobility of O-Arab on this medium is more readily apparent in the absence of haemoglobin S (see Fig. 5.44).

Sickle cell/haemoglobin Lepore

Compound heterozygosity for sickle cell haemoglobin and haemoglobin Lepore-Boston [333, 334] has been reported in Mediterranean (Greek and Italian), African Caribbean, African American and Indian populations. Sickle cell/haemoglobin Lepore leads to sickle cell disease of variable severity, but resembling sickle cell/ β thalassaemia more closely than sickle cell anaemia. Of 10 cases reported up to 1997 three were severe and seven were mild [334].

Fig. 4.54 HPLC chromatogram (Bio-Rad variant II) in a patient with haemoglobin S/O-Arab compound heterozygosity; the haemoglobin O-Arab in this and another patient had retention times on a Bio-Rad Variant II of 4.89 and 4.90 minutes respectively; peaks, from left to right, are altered F, F₀ (shaded), altered S (double peak), A₂ (shaded), S and O-Arab. Note that the S peak is flanked by two small peaks that represent altered O-Arab.

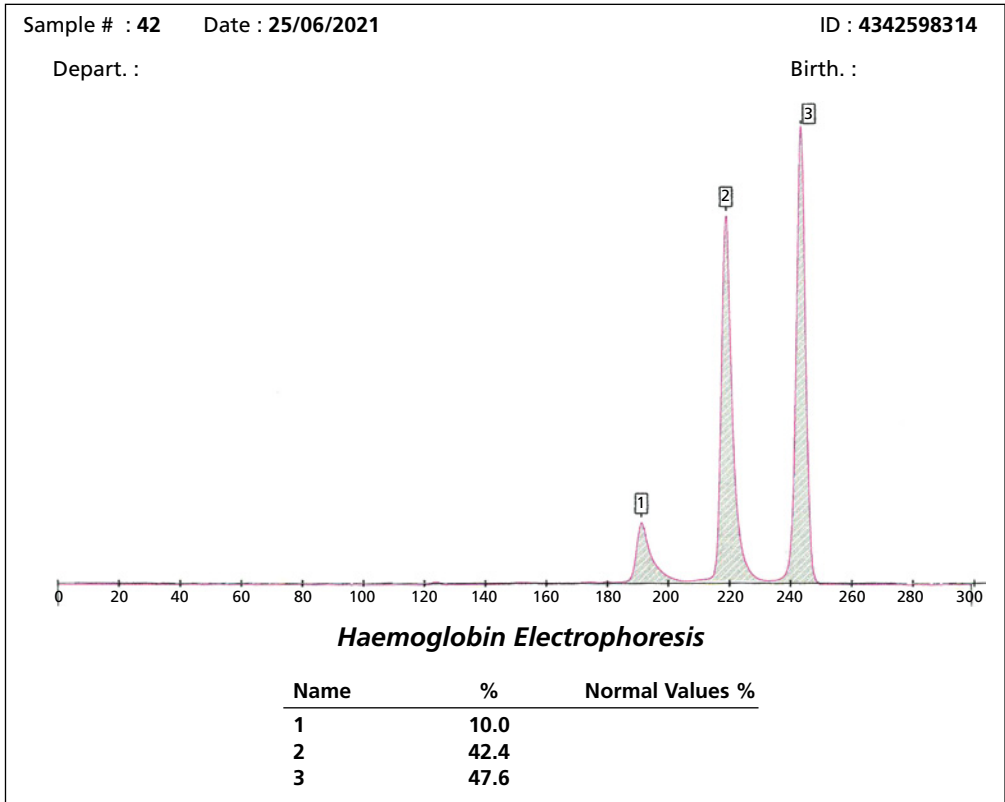
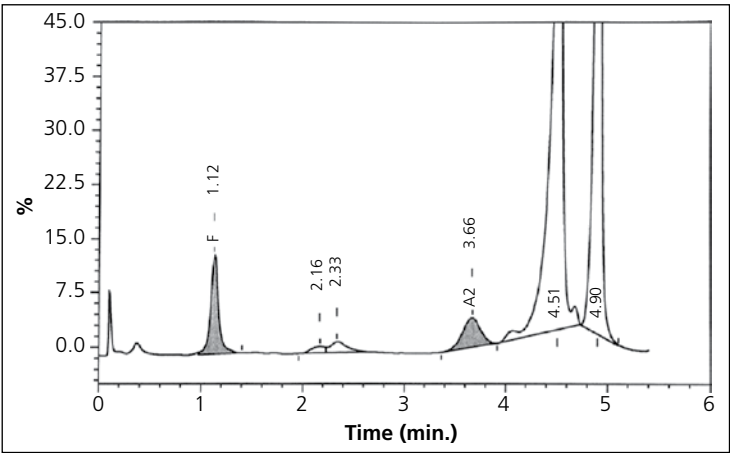


Fig. 4.55 Capillary electrophoresis (Sebia Capillarys) in a patient with haemoglobin S/O-Arab compound heterozygosity. The peaks from left to right are haemoglobins F, S and O-Arab.

Table 4.11 Making a distinction between haemoglobin O-Arab and haemoglobin C-Harlem.

	Haemoglobin O-Arab	Haemoglobin C-Harlem
Frequency	Uncommon	Rare
Clinical severity of compound heterozygous state with haemoglobin S	As severe as sickle cell anaemia	Somewhat milder than sickle cell anaemia
Sickle solubility test	Negative	Positive
Mobility on cellulose acetate electrophoresis at alkaline pH	Mobility of C	Mobility of C
Mobility on agarose gel electrophoresis at acid pH	Slightly slower than S (i.e. slightly towards C)	With S
Mobility on citrate agar electrophoresis at acid pH	Somewhat faster than S (i.e. slightly on the A side of S)	With S
High performance liquid chromatography	Between S and C	Between S and C
Isoelectric focusing	With E	With E

Haematological variables reported in adults [13, 273, 334] have been Hb 80–133 g/l, MCV 66.5–83 fl, MCH 24.3–27.6 pg and reticulocyte count 3–13% (33% in one case). The blood film shows anisocytosis, hypochromia, microcytosis and some sickle cells.

Since haemoglobin Lepore has the same mobility as haemoglobin S on electrophoresis at alkaline pH the only bands apparently present are haemoglobins F, S and A₂ and diagnostic confusion with sickle cell anaemia and sickle cell/ $\delta\beta^0$ thalassaemia can therefore occur. However, other techniques such as HPLC (Fig. 4.56) show that haemoglobin Lepore is usually around 10–12% of total haemoglobin (20% in one case) while haemoglobin S is 63–90% and haemoglobin F 5–25%. Electrophoresis at acid pH shows two bands, one with the mobility of haemoglobin A, which represents the haemoglobin Lepore. The proportion of haemoglobin A₂ is variable, having been reported to be reduced, normal or slightly elevated in different cases (0.9–4%) [13, 334].

Sickle cell/ $\delta\beta^0$ thalassaemia

Sickle cell/ $\delta\beta^0$ thalassaemia has been observed in Mediterranean populations (Greek, Sicilian, other Italian) and in Arabs, Indians and African Americans [13, 273, 335]. This compound

heterozygous state is generally, but not always [335], much milder than sickle cell anaemia because the high percentage of haemoglobin F protects against sickling. There is mild anaemia and splenomegaly.

The blood count shows an Hb of around 100–120 g/l and an MCV that is slightly reduced (76–83 fl). The reticulocyte count is mildly elevated, usually 2–4%. The blood film shows anisocytosis, poikilocytosis and hypochromia. Haemoglobin S is the major haemoglobin component with haemoglobin F being 15–50% of total haemoglobin. The proportion of haemoglobin A₂ is normal or low (1.5–3.1%). Sickle cell/ $\delta\beta^0$ thalassaemia differs from microcytic cases of sickle cell anaemia, having a higher Hb, lower reticulocyte count and lower haemoglobin A₂ percentage (see Table 4.8). However, definitive diagnosis requires DNA analysis.

Sickle cell/hereditary persistence of fetal haemoglobin

Compound heterozygosity for haemoglobin S and deletional or pancellular HPFH is either asymptomatic or produces quite mild sickle cell disease [273]. Haemoglobin S/HPFH has been reported in Africans, African Caribbeans and African Americans. There can be mild haemolytic anaemia and splenomegaly or

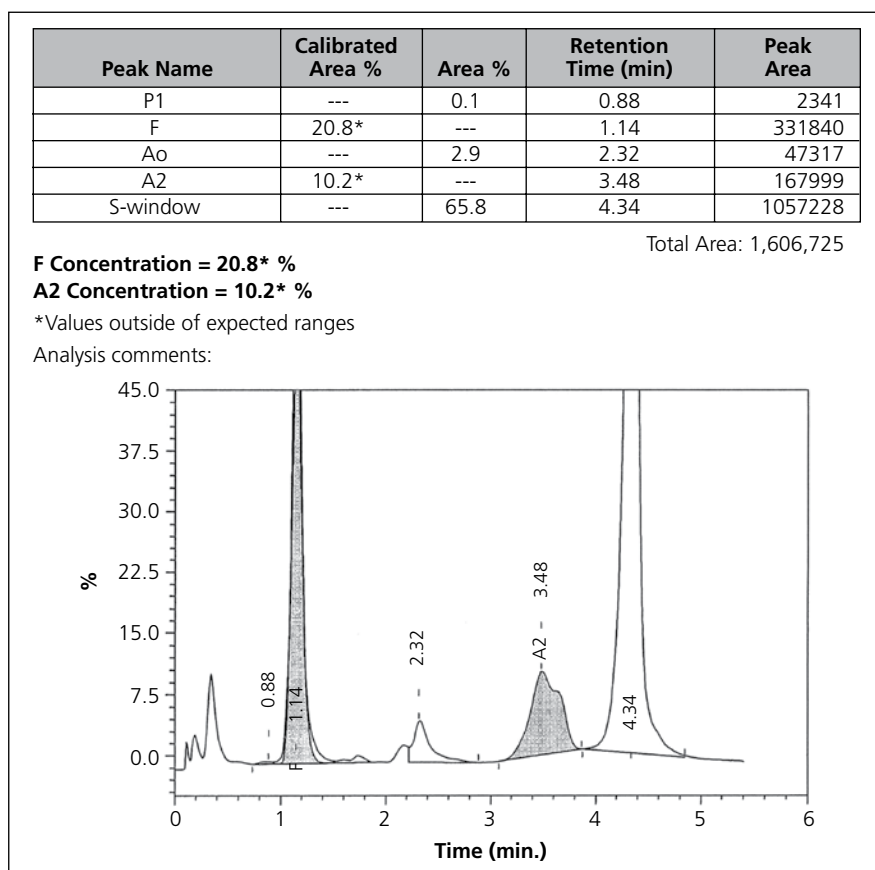


Fig. 4.56 HPLC chromatogram (Bio-Rad variant II) in a patient with compound heterozygosity for haemoglobin S and haemoglobin Lepore showing triple peak of post-translationally modified haemoglobin F, haemoglobin F₀ (shaded), altered haemoglobin Lepore and S in the A₀ window, haemoglobin A₂ plus Lepore (shaded) and haemoglobin S.

minor clinical features consequent on sickling. The Hb and reticulocyte count are usually normal but microcytosis is common and occasionally there is mild anaemia and reticulocytosis of 2–4%. The blood film (Fig. 4.57) may show anisocytosis, microcytosis and target cells. Haemoglobin electrophoresis or HPLC shows haemoglobin F of 15–35% (usually 20–30%), haemoglobin A₂ which is low normal or slightly reduced and haemoglobin S comprising around 60–80% of total haemoglobin. Virtually all cells are F cells [267]. Haemoglobin A is absent. In infancy, haemoglobin F is 50–90% with a decline and stabilisation at adult levels by 3–5 years [336]. The proportions of various haemoglobins in S/HPFH are similar to those of

S/ β^0 thalassaemia. Distinction between the two is aided by the usual lack of symptoms and by the fact that the Hb and reticulocyte count are often normal in S/HPFH (see Table 4.8). Definitive diagnosis requires family studies or DNA analysis, which most commonly shows the HPFH-2 deletion that is particularly common in Ghana.

Sickle cell/haemoglobin E compound heterozygosity

Compound heterozygosity for haemoglobin S and haemoglobin E produces a condition that is usually either asymptomatic or clinically mild, similar to the mild forms of haemoglobin

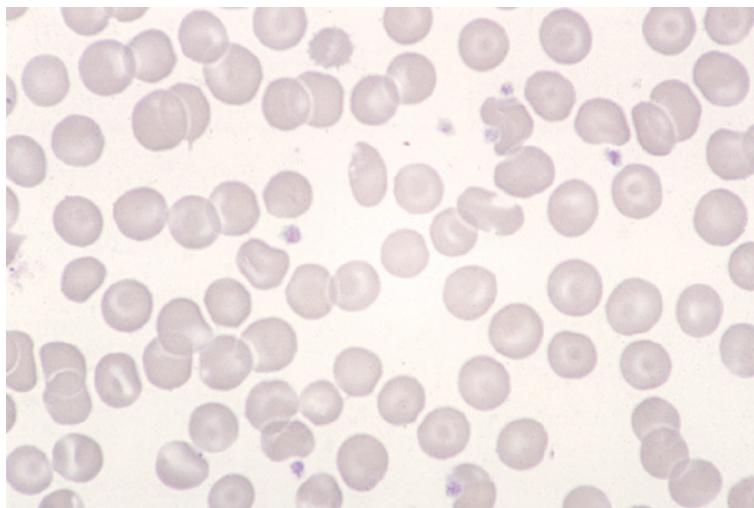


Fig. 4.57 Blood film in sickle cell/hereditary persistence of fetal haemoglobin compound heterozygosity showing mild poikilocytosis and target cell formation. MGG $\times 100$.

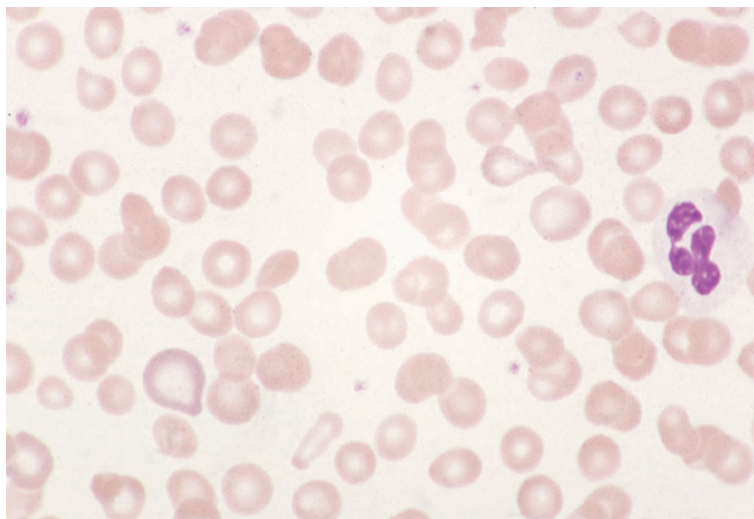


Fig. 4.58 Blood film in sickle cell/haemoglobin E compound heterozygosity showing microcytosis and poikilocytosis. (With thanks to Dr Rajeev Gupta.) MGG $\times 100$.

S/ β^+ thalassaemia [13, 273, 337–344]. Sickle cell/haemoglobin E has been observed in Turks, African Americans, African Caribbeans, Indians, Pakistanis, Haitians, in the Middle East (including Saudi Arabia and Oman), in South-East Asia and increasingly in individuals of mixed ancestry. There are sometimes complications related to sickling, particularly in adults; these have included painful crises, acute chest syndrome, splenomegaly, splenic sequestration (which can occur in adults), splenic infarction (recurrent during flights in one patient), hepatomegaly, osteonecrosis and retinal disease [338, 341, 345–347]. Death from

embolisation of infarcted bone marrow, associated with parvovirus B19 infection, has been reported in a child [347]. There can be mild haemolysis (often compensated), jaundice and gallstones. The Hb is often normal but may be reduced (80–146 g/l) with sometimes a slight increase in the reticulocyte count (1.5–5.3%). The MCV can be normal but is usually reduced (68–97 fl in adults). The blood film (Fig. 4.58) can show target cells which are sometimes numerous. Sickle cells have been observed [337] but this is not usual. Haemoglobin S is a larger proportion of total haemoglobin than haemoglobin E (e.g.

55–63%) with around 33% of haemoglobin E plus A₂ and 0–7.5% haemoglobin F [326, 338–341, 343] (Fig. 4.59). HPLC (Bio-Rad Variant II) shows two major peaks, one representing haemoglobin E plus A₂ and the other haemoglobin S (Fig. 4.60); on other HPLC instruments, haemoglobins E and A₂ may form separate peaks. On capillary electrophoresis, there are two major peaks, haemoglobins E and S, plus a haemoglobin A₂ peak (Fig. 4.61). With both techniques it is apparent that haemoglobin E (with or without haemoglobin A₂) is a much lower percentage than haemoglobin S.

Sickle cell/ $\delta^0\beta^+$ thalassaemia

Sickle cell/ $\delta^0\beta^+$ thalassaemia, as the result of formation of a $\delta\beta$ fusion gene, was reported in four brothers in a Senegalese family. Hb varied from 109 to 134 g/l, MCV from 76 to 85 fl, haemoglobin S from 58% to 70%, haemoglobin A from 12% to 16% and haemoglobin F from 12% to 30% [348]. The proband was asymptomatic.

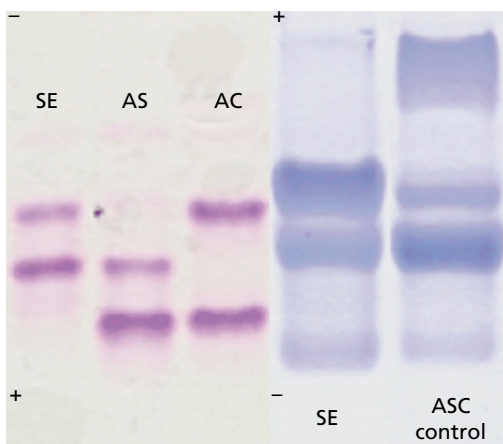


Fig. 4.59 Haemoglobin electrophoresis at acid and alkaline pH in sickle cell/haemoglobin E compound heterozygosity; the first three lanes from the left are cellulose acetate electrophoresis at alkaline pH; the two lanes on the right are agarose gel electrophoresis at acid pH with the ASC control showing, from below up, haemoglobins A, S and C. (With thanks to Dr Rajeev Gupta, Mr Martin Jarvis and Dr Anne Yardumian.)

Other rare compound heterozygous and related states

Compound heterozygosity for haemoglobin C and haemoglobin C-Harlem appears to produce a much milder disease than sickle cell/haemoglobin C disease. One reported patient who presented with haematuria had anaemia and splenomegaly but no symptoms suggestive of sickling [11]; the blood film showed many target cells and occasional sickle cells. Compound heterozygosity for haemoglobin S and haemoglobin S-Antilles produces a very severe form of sickle cell disease [43]. Compound heterozygosity for haemoglobin S and haemoglobin S-Oman has been described, presenting at the age of one year [349]; it is likely that the phenotype would be severe since this double substitution haemoglobin can cause disease in heterozygotes. Compound heterozygosity for haemoglobin S and the electrophoretically silent variant, haemoglobin Quebec-Chori, causes sickle cell disease [53]. Compound heterozygosity for haemoglobin S and the electrophoretically silent unstable variant, haemoglobin Volga, caused splenic sequestration and mild disease thereafter in one patient [350]. Compound heterozygosity for haemoglobin S and mildly unstable haemoglobins such as haemoglobin Hope and haemoglobin Siriraj (Figs 4.62 and 4.63) can cause mild haemolysis [273, 351]. A compound heterozygote for haemoglobin S and the mildly unstable variant, haemoglobin Tyne, had the clinical features of sickle cell disease [352]. A compound heterozygote for S and haemoglobin Hofu (a fast-moving haemoglobin) had a significant anaemia (Hb 96 g/l), 73% haemoglobin S and apparently clinical features of sickling [326]. Compound heterozygosity for haemoglobin S and the $\Lambda\gamma\beta$ fusion haemoglobin, haemoglobin Kenya, has an interestingly mild phenotype, given that S is 60–70% of total haemoglobin [353]. Compound heterozygotes appear to be generally asymptomatic with a mild microcytic anaemia [131, 354]. Haemoglobin Kenya is about 18% and haemoglobin F around 8–10% with a pancellular distribution [131, 354] (Fig. 4.64a,b);

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.92	1102
F	0.6	---	1.07	11956
Unknown	---	0.2	1.19	3848
P2	---	0.6	1.30	11649
P3	---	1.4	1.88	26586
Ao	---	6.6	2.35	126228
A2	37.1*	---	3.72	678089
S-window	---	55.0	4.39	1051942

Total Area: 1,911,400

F Concentration = 0.6 %

A2 Concentration = 37.1* %

*Values outside of expected ranges

Analysis comments:

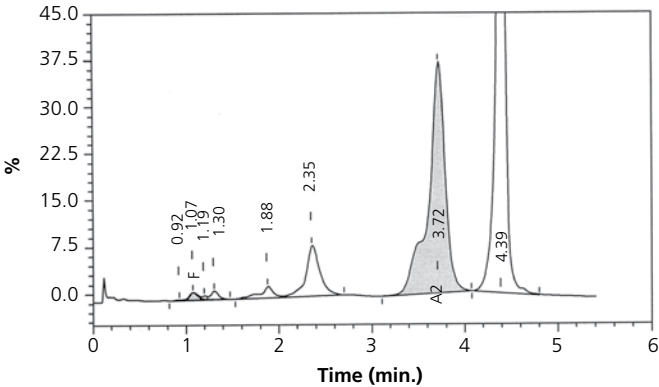


Fig. 4.60 HPLC (Bio-Rad Variant II) in sickle cell/ haemoglobin E compound heterozygosity. The peaks from left to right are: injection artefact, haemoglobin F (shaded), three peaks representing post-translationally modified haemoglobin S and A, a complex peak representing haemoglobin A₂ plus E (shaded) and haemoglobin S.

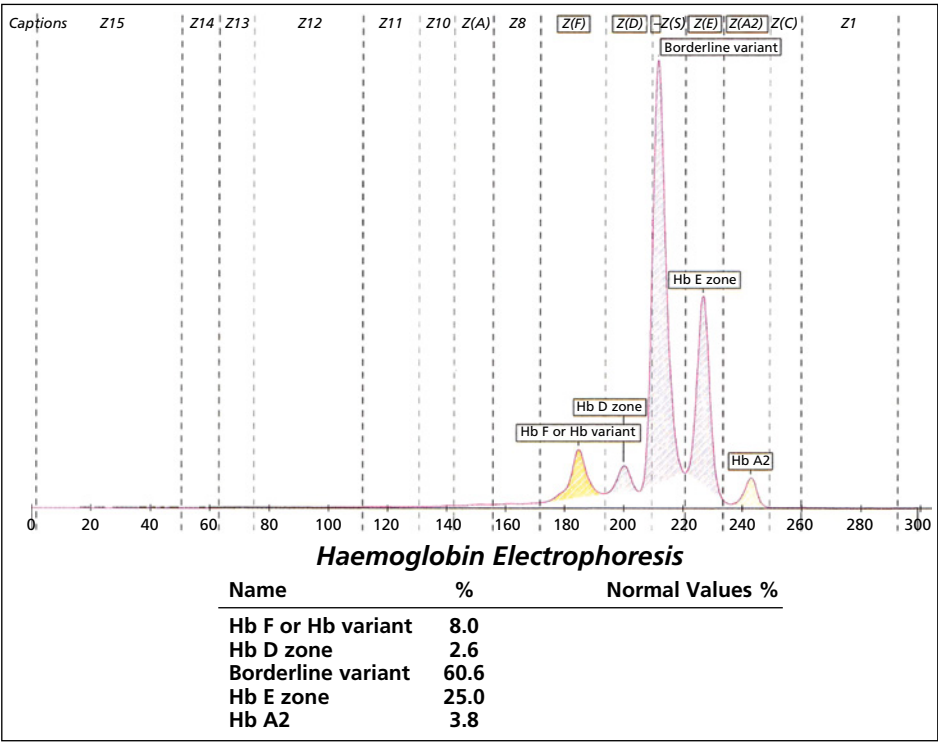
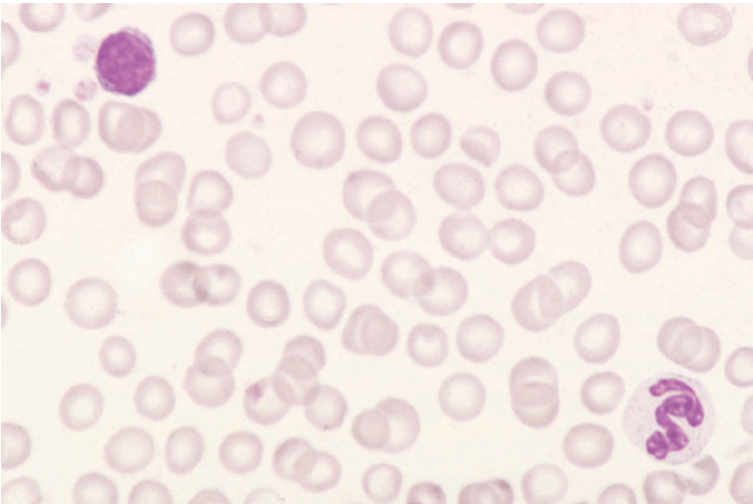


Fig. 4.61 Capillary electrophoresis in sickle cell/haemoglobin E compound heterozygosity. The peaks from left to right are haemoglobin F, unidentified, haemoglobin S, haemoglobin E and haemoglobin A₂.

Fig. 4.62 Blood film of a one-year-old child with haemoglobin S/haemoglobin Siriraj compound heterozygosity showing anisocytosis and hypochromia. The red cell indices were RBC $4.87 \times 10^{12}/l$, Hb 112 g/l, MCV 67 fl, MCH 23.3 pg and MCHC 344 g/l. MGG $\times 100$.



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.9*	---	1.09	31471
Ao	---	3.4	2.31	54172
A2	9.1*	---	3.65	130089
S-window	---	58.8	4.51	940293
Unkown	---	0.5	4.89	8749
C-window	---	27.1	5.07	433850

Total Area: 1,598,624

F Concentration = 1.9* %

A2 Concentration = 9.1* %

*Values outside of expected ranges

Analysis comments:

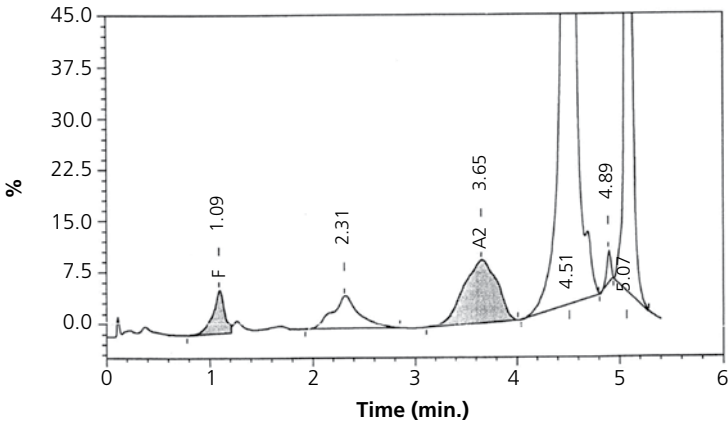


Fig. 4.63 HPLC chromatogram (Bio-Rad Variant II) in a patient with compound heterozygosity for haemoglobin S and haemoglobin Siriraj showing low peaks of altered haemoglobin F, haemoglobin F₀ (shaded), glycated haemoglobin S in the A₀ window, haemoglobin A₂ plus other post-translationally modified haemoglobin S (shaded), haemoglobin S, and haemoglobin Siriraj in the haemoglobin C window. Note the two small sharp peaks between the peaks for haemoglobins S and Siriraj, which represent post-translationally modified haemoglobin Siriraj.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.85	2246
F	11.2*	---	1.13	458790
P3	---	3.0	1.78	122888
Unknown	---	1.2	2.04	50400
Ao	---	2.5	2.18	101135
Unknown	---	0.1	2.46	2798
A2	22.3*	---	3.48	850031
S-window	---	61.0	4.29	2484524

Total Area: 4,072,812*

F Concentration = 11.2* %

A2 Concentration = 22.3* %

*Values outside of expected ranges

Analysis comments:

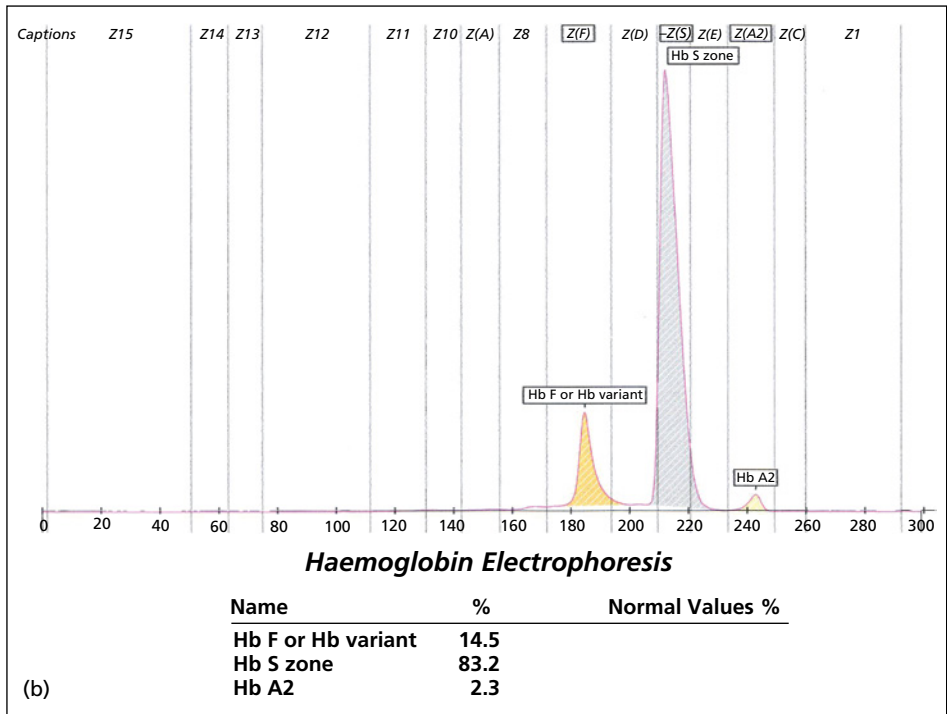
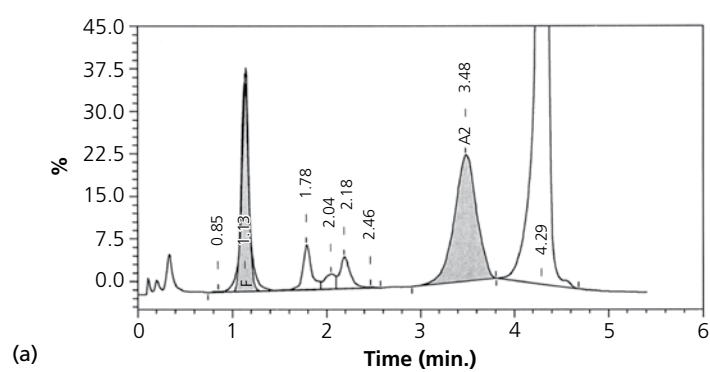


Fig. 4.64 Investigations of a patient with compound heterozygosity for haemoglobin S and haemoglobin Kenya: (a) HPLC chromatogram (Bio-Rad Variant II) showing major peaks, from left to right, representing haemoglobin F, haemoglobin Kenya plus haemoglobin A₂, and haemoglobin S; complex minor peaks are post-translationally modified haemoglobins; (b) capillary electrophoresis (Sebia Capillaries) with peaks from left to right being haemoglobin F, haemoglobin S plus Kenya and haemoglobin A₂.

haemoglobins F and A₂ will inhibit sickling and one might be tempted to postulate that this could also be true of haemoglobin Kenya. Splenic infarction and chest crisis can occur in compound heterozygotes for haemoglobin S and haemoglobin New York, a mildly unstable haemoglobin with low oxygen affinity, which has the same retention time as haemoglobin S on HPLC [355]. Compound heterozygosity for haemoglobin S and haemoglobin Monroe leads to a clinical syndrome resembling haemoglobin S/ β^0 thalassaemia, since haemoglobin Monroe is unstable and constitutes only about 2% of total haemoglobin [356]. Compound heterozygosity for haemoglobin S and various other variant haemoglobins can cause haematological abnormalities as a result of the characteristics of the second variant, rather than as a result of any interaction between the two variant haemoglobins; this appears to be true of haemoglobins I-Toulouse (unstable), San Diego (high affinity), Shelby (mildly unstable), Lufkin (mildly unstable), Hope (unstable and low oxygen affinity) and North Shore ('thalassaemic') [133].

Two individuals have been reported with inheritance of β^S from one parent and β from the other but with post-zygotic mitotic recombination leading to mosaic isodisomy and a mixture of AS and SS cells; this led to late presentation with a sickle cell disease phenotype [85].

The vast majority of β chain variants do not interact with haemoglobin S, so that compound heterozygotes have clinical and haematological features that resemble those of sickle cell trait. These include haemoglobins Camden, Caribbean, D-Ouled Rabah, D-Ibadan, Detroit, E-Saskatoon, G-Galveston, G-San Jose, G-Szuhu, J-Amiens, J-Baltimore, J-Bangkok, K-Ibadan, K-Matupo, K-Woolwich, Mobile, N-Baltimore, Ocho-Rios, Osu-Christiansborg, Pyrgos and Richmond [161, 218].

Sickle cell disease in heterozygotes

Three variant haemoglobins in which the $\beta^{6\text{Glu} \rightarrow \text{Val}}$ substitution is one of two substitutions are

capable of producing sickle cell disease in heterozygotes. They are haemoglobin S-Antilles, haemoglobin S-Oman and haemoglobin Jamaica Plain (see Table 4.2).

Haemoglobin S-Oman is usually coinherited with either $-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$ and microcytosis is therefore common [357]. Heterozygotes have from 4% to 25% haemoglobin S-Oman with the percentage tending to be lower in those with $-\alpha/-\alpha$ [357]. Disease in heterozygotes varies from mild to severe. Strangely, severe disease is more likely in those with a *higher* haemoglobin F level; severity tends to be less in those with $-\alpha/-\alpha$ than in those with $-\alpha/\alpha\alpha$ but does not show a close relationship to the percentage of the variant haemoglobin [357]. The morphology of sickle cells in patients who are simple or compound heterozygotes for haemoglobin S-Oman differs from the morphology of classic sickle cells. There are cells that are pointed at both ends but fat in the middle; they have been compared to a yarn/knitting needle or to Napoleon hats [349] (Fig. 4.65). Affected heterozygotes also differ clinically from sickle cell anaemia in that splenomegaly can persist into adult life. The variant haemoglobin can be detected by HPLC and capillary electrophoresis (Fig. 4.66a,b).

Sickle cell disease can also occur in heterozygotes if there is coinheritance of another condition leading to a high concentration of 2,3-DPG and reduced oxygen affinity. For example, a patient who had coinherited a severe pyruvate kinase deficiency had a two-fold increase in 2,3-DPG leading to reduced oxygen affinity and symptomatic sickling crises [55].

Sickle cell disease resulting from uniparental disomy

A unique patient has been described with sickle cell disease resulting from post-zygotic mitotic recombination leading to uniparental disomy [358]. The child had 52% haemoglobin S and a mixture of AS and SS cells.

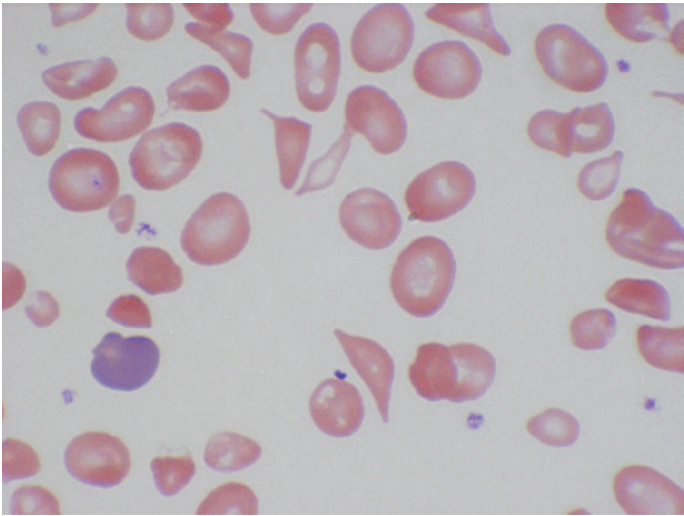


Fig. 4.65 Blood film in a patient with compound heterozygosity for haemoglobin S and haemoglobin S-Oman compound heterozygosity showing the 'Napoleon hat' red cells that are characteristic of haemoglobin S-Oman. (With thanks to Dr Samir Al Azzawi, Muscat, Sultanate of Oman.)

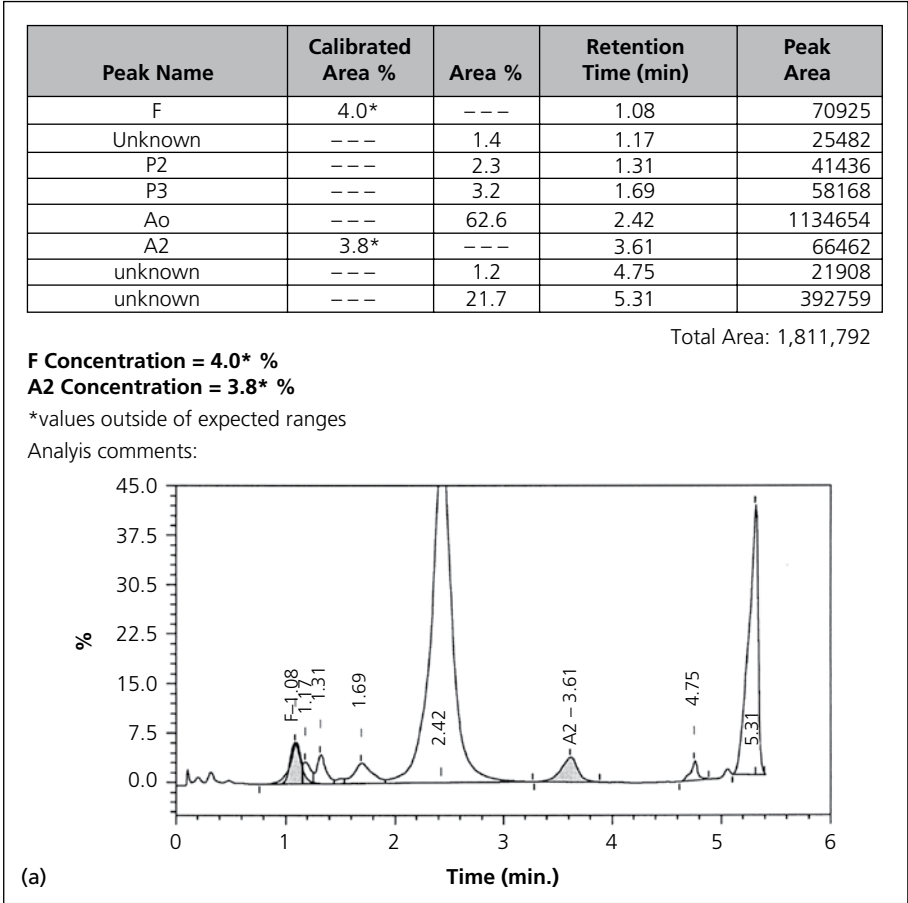


Fig. 4.66 Haemoglobin S-Oman heterozygosity: (a) HPLC (Bio-Rad Variant II) the peaks from left to right being haemoglobins F, glycated A, other post-translationally modified A, A_o, A₂, post-translationally modified S-Oman (two small peaks) and S-Oman; (b) capillary electrophoresis (Sebia Capillars), the peaks from left to right being haemoglobins A, F, A₂ and S-Oman. (Continued on p. 281.)

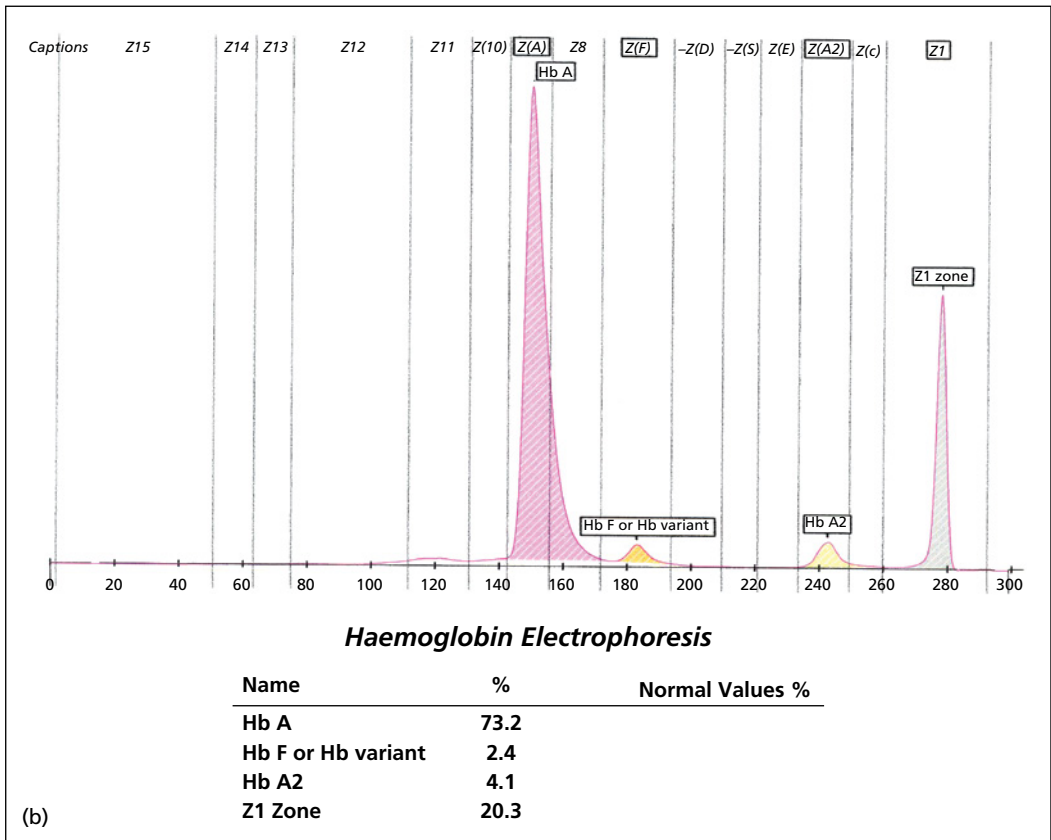


Fig. 4.66 Continued.

Check your knowledge

One to five answers may be correct. Answers to most questions can be either found in this chapter or deduced from information given. Answers are given on page 298.

- 4.1 The coinheritance of haemoglobin S and the following haemoglobins usually produces a clinically significant sickling disorder
- haemoglobin C
 - haemoglobin G-Philadelphia
 - haemoglobin D-Punjab
 - haemoglobin Lepore
 - haemoglobin A
- 4.2 Haemoglobin S occurs in a significant proportion of individuals of the following ethnic groups
- Australian aboriginals
 - Greeks
 - Southern Italians and Sicilians
 - Saudi Arabs
 - Nigerians
- 4.3 Recognised features of sickle cell trait include
- a defect in urine concentrating ability
 - an increased incidence of gallstones
 - an increased reticulocyte count
 - leg ulcers
 - susceptibility to clinically significant sickling in conditions of severe hypoxia
- 4.4 The following variant haemoglobins have the same mobility as haemoglobin S on cellulose acetate electrophoresis at alkaline pH
- haemoglobin C
 - haemoglobin D
 - haemoglobin E

- (d) haemoglobin F
 - (e) haemoglobin G
- 4.5 The likelihood of red cell sickling occurring is increased by
- (a) acidosis
 - (b) a lower partial pressure of oxygen
 - (c) an increased percentage of haemoglobin F
 - (d) reduced blood flow through tissues
 - (e) a lower mean cell haemoglobin concentration (MCHC)
- 4.6 In comparison with sickle cell anaemia, patients with compound heterozygosity for haemoglobin S and haemoglobin C usually have
- (a) a higher percentage of haemoglobin A
 - (b) more severe anaemia
 - (c) a higher incidence of proliferative retinopathy
 - (d) a higher incidence of avascular necrosis of the femoral head
 - (e) earlier onset of blood film features of hyposplenism
- 4.7 Significant disease would be predicted in 25% of offspring if the partner of a pregnant woman with sickle cell trait had
- (a) α thalassaemia trait
 - (b) β thalassaemia trait
 - (c) δ thalassaemia trait
 - (d) $\delta\beta$ thalassaemia trait
 - (e) γ thalassaemia trait
- 4.8 The disease phenotype is usually appreciably less severe than that of homozygosity for haemoglobin S in
- (a) sickle cell/haemoglobin C disease
 - (b) sickle cell/ β^+ thalassaemia
 - (c) sickle cell/deletional hereditary persistence of fetal haemoglobin
 - (d) sickle cell/ $\delta\beta^0$ thalassaemia
 - (e) sickle cell/haemoglobin E
- 4.9 On haemoglobin electrophoresis at alkaline pH homozygosity for haemoglobin S cannot be distinguished from
- (a) sickle cell/haemoglobin C disease
 - (b) sickle cell/ β^0 thalassaemia
 - (c) sickle cell/haemoglobin D-Punjab
 - (d) sickle cell/haemoglobin Lepore
 - (e) heterozygosity for both haemoglobin S and haemoglobin G-Philadelphia
- 4.10 A higher mortality rate in sickle cell anaemia correlates with
- (a) higher white cell count
 - (b) coexisting α thalassaemia trait
 - (c) lower percentage of haemoglobin F
 - (d) male gender
 - (e) previous cerebrovascular accident
- 4.11 The blood count in sickle cell/haemoglobin C disease is characterised by
- (a) generally mild anaemia
 - (b) reticulocytosis
 - (c) increased mean cell volume (MCV)
 - (d) increased mean cell haemoglobin concentration (MCHC)
 - (e) increased red cell distribution width (RDW) and haemoglobin distribution width (HDW)
- 4.12 The haemoglobin F percentage in sickle cell anaemia is affected by
- (a) age
 - (b) gender
 - (c) haplotype of the β globin gene cluster
 - (d) crizanlizumab therapy
 - (e) hydroxycarbamide (hydroxyurea) therapy
- 4.8 The disease phenotype is usually appreciably less severe than that of homozygosity for haemoglobin S in
- (a) sickle cell/haemoglobin C disease
 - (b) sickle cell/ β^+ thalassaemia
 - (c) sickle cell/deletional hereditary persistence of fetal haemoglobin
 - (d) sickle cell/ $\delta\beta^0$ thalassaemia
 - (e) sickle cell/haemoglobin E
- 4.9 On haemoglobin electrophoresis at alkaline pH homozygosity for haemoglobin S cannot be distinguished from
- (a) sickle cell/haemoglobin C disease
 - (b) sickle cell/ β^0 thalassaemia

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Answers to questions

- | | | | | | |
|---|---|---|---|--|--|
| 4.1 (a) T
(b) F
(c) T
(d) T
(e) F | 4.3 (a) T
(b) F
(c) F
(d) F
(e) T | 4.5 (a) T
(b) T
(c) F
(d) T
(e) F | 4.7 (a) F
(b) T
(c) F
(d) T
(e) F | 4.9 (a) F
(b) T
(c) T
(d) T
(e) F | 4.11 (a) T
(b) T
(c) F
(d) T
(e) T |
| 4.2 (a) F
(b) T
(c) T
(d) T
(e) T | 4.4 (a) F
(b) T
(c) F
(d) F
(e) T | 4.6 (a) F
(b) F
(c) T
(d) T
(e) F | 4.8 (a) T
(b) T
(c) T
(d) T
(e) T | 4.10 (a) T
(b) F
(c) T
(d) T
(e) T | 4.12 (a) T
(b) T
(c) T
(d) F
(e) T |

5 Other significant haemoglobinopathies

Variant haemoglobins may be clinically significant but many are clinically silent. The recognition of those that are clinically silent can nevertheless be important in the diagnostic laboratory since their presence can lead to diagnostic confusion. Problems of two types can arise. Clinically irrelevant variant haemoglobins can be confused with clinically significant variants because of a similar electrophoretic mobility or high performance liquid chromatography (HPLC) retention time. In addition, there can be coinheritance of two variants, leading to the presence of multiple bands or peaks that can be difficult to interpret. This chapter will therefore deal both with clinically relevant haemoglobinopathies and with other variant haemoglobins that can cause diagnostic confusion.

More than 1000 variant haemoglobins have been described. The majority of recognised variant haemoglobins are α or β chain variants. A variant α chain leads to variant forms of haemoglobins A, A₂ and F. A variant β chain leads to a variant of haemoglobin A. δ and γ chain variants also occur. There is no reason to doubt the existence of variant ϵ and ζ chain variants. Functional abnormalities of haemoglobin that can result from mutations in globin genes are shown in Table 5.1.

It should be noted that, although low oxygen affinity haemoglobins lead to anaemia and sometimes cyanosis, there are usually no symptoms of anaemia as there is normal oxygen delivery to tissues. The anaemia is the result of a reduced erythropoietic drive.

The variant haemoglobins that are of diagnostic but not clinical significance are mainly haemoglobins with the mobility of either S or C/E on cellulose acetate electrophoresis at alkaline pH

or on capillary electrophoresis. There are also other, less common, variant haemoglobins that are diagnostically important because they have similar retention times to S, C, E, D-Punjab or glycosylated haemoglobin A on HPLC. If cellulose acetate electrophoresis is the primary method, it is important to distinguish haemoglobins such as haemoglobin G-Philadelphia or haemoglobin D-Iran, which are not clinically important, from haemoglobin D-Punjab/D-Los Angeles, which is of importance because of its interaction with haemoglobin S. If HPLC is the primary method the uncommon variant haemoglobins that have a similar retention time to clinically important variants must similarly be distinguished from each other (e.g. haemoglobin E from haemoglobin Lepore and haemoglobin D-Punjab from haemoglobin G-Philadelphia) (see Table 2.3).

A β chain variant would be expected to comprise about 50% of total haemoglobin. However, if the abnormal β chain is synthesised at a reduced rate or if there is preferential combination of α chains with the normal rather than the variant β chain, the proportion will be less. Among the variant β chains synthesised at a considerably reduced rate is β^E with the result that the proportion of haemoglobin E in heterozygotes does not usually exceed 25–30%. Variant chains with a reduced affinity for α chain compared to β^A include β^S and β^C , probably because they are more electropositive than normal β chain [1, 2]; as a result of the reduced affinity, the percentage of the variant is somewhat less than 50%. The converse is seen with variant β chains, including $\beta^{J\text{-Baltimore}}$ and $\beta^{J\text{-Iran}}$ that are more electronegative [1, 2] and have a greater affinity than normal β chain for α chains; the percentage of the variant is therefore greater than 50%.

Table 5.1 The types of functional abnormality that can occur as a result of mutations in globin genes.

Functional abnormality	Example
Polymerisation leading to sickle cell formation	Haemoglobin S Haemoglobin C-Harlem and other double substitution haemoglobins
Interaction with haemoglobin S, permitting sickling in compound heterozygotes	Haemoglobin D-Punjab/D-Los Angeles Haemoglobin C Haemoglobin O-Arab
Reduced solubility leading to crystal formation and haemolytic anaemia	Haemoglobin C
Increased oxygen affinity leading to polycythaemia	Haemoglobin Chesapeake Haemoglobin Kempsey
Reduced oxygen affinity leading to anaemia	Haemoglobin S Haemoglobin Kansas
Haemoglobin instability leading to a Heinz body haemolytic anaemia	Haemoglobin Köln
Extreme instability leading to a thalassaemic phenotype	Haemoglobin Terre Haute
Reduced rate of synthesis leading to a thalassaemic phenotype	Haemoglobin Lepore Haemoglobin E
Increased tendency to oxidation leading to methaemoglobin formation and cyanosis	Haemoglobin M-Saskatoon Haemoglobin M-Hyde Park

When there is coexisting α thalassaemia, a positively charged variant chain such as β^S or β^C competes less well than normal β chain for the reduced number of α chains so that the percentage of the variant is lower than in individuals with a full complement of α genes. The converse is seen with variants such as J-Baltimore when the negatively charged variant chain is more able to compete for the reduced pool of α chains and the variant is present in an even higher percentage [1].

Predicting the percentage of an α chain variant is more complex since not only are there four α genes but the $\alpha 2$ gene is transcribed at a faster rate than the $\alpha 1$ globin gene. The two allelic $\alpha 2$ genes normally contribute between them about 75% of α chains. An α chain variant would therefore be expected to comprise either about 37.5% or about 12.5% of total haemoglobin. Proportions may differ if: (i) the variant α chain is synthesised at a reduced rate; (ii) the variant α chain shows a greater or lesser affinity for β chain than does the normal α chain; (iii) the variant haemoglobin is

also unstable; or (iv) there is coexisting α thalassaemia. As for β globin variants, charge may influence the affinity of the variant α chain for the normal β chain. For example $\alpha^{M-Iwate}$, which is more electropositive than normal α chain, combines preferentially with electronegative β chains so that haemoglobin M-Iwate comprises 22–27% of total haemoglobin, even though it is an $\alpha 1$ variant [2].

Haemoglobin G-Philadelphia illustrates the complexity of the interaction between an α chain variant and α thalassaemia. The $\alpha^{G-Philadelphia}$ gene can occur either as one of two α genes on a chromosome ($\alpha^G\alpha$) or as the only α gene on a chromosome ($-\alpha^G$) as a result of the mutation having occurred in a fusion $\alpha 2\alpha 1$ gene on a chromosome with a 3.7 kb deletion. The former mutation would be expected to lead to the variant being about 12.5% of total haemoglobin and the latter to the variant being somewhat more than a third of total haemoglobin. A further complicating factor is that the same ethnic group may have both haemoglobin

G-Philadelphia and a high prevalence of unlinked α thalassaemia. There may then be α thalassaemia in *trans* to the variant α gene, giving the genotype $-\alpha^G/-\alpha$ with haemoglobin G-Philadelphia being about 45% of total haemoglobin.

Haemoglobin C

Haemoglobin C is a variant haemoglobin with a mutation in the β globin gene at the same site as the mutation in the gene encoding β^S . It was first recognised by Itano and Neel in 1950 [3]. Its structure is $\alpha_2\beta_2^{7\text{Glu}\rightarrow\text{Lys}}$. It can be present in the heterozygous state (haemoglobin C trait or carrier), in the homozygous state (haemoglobin C disease) and in a variety of compound heterozygous states such as sickle cell/haemoglobin C disease and haemoglobin C/ β thalassaemia. Sickle cell/haemoglobin C disease has been discussed in Chapter 4. Other haemoglobinopathies with haemoglobin C will be discussed in this chapter.

Haemoglobin C is thought to have originated in West Africa, west of the Niger River (Fig. 5.1) (see Table 4.1). In northern Ghana the proportion of individuals with haemoglobin C is as high as 40% and in northern Ivory Coast up to 50%. In Burkina Faso it is 15–40%. It would appear that haemoglobin C arose in the region spanning Burkina Faso and the Ivory Coast and Ghana. It is found in individuals of African descent in the Caribbean (3.5% prevalence), USA (2% prevalence among African Americans), Canada and the UK. A high incidence has been noted in a Bedouin tribe in northern Israel [4]. There is also a significant incidence of haemoglobin C in North Africa and a low incidence in southern Europe (Spain and Sicily). However, it should be noted that some early reports of the presence of haemoglobin C, based only on electrophoresis at alkaline pH, may have been a misidentification of haemoglobin O-Arab as haemoglobin C. Haemoglobin C appears to have had an independent origin in Oman and Thailand [5, 6]. Two cases have been reported

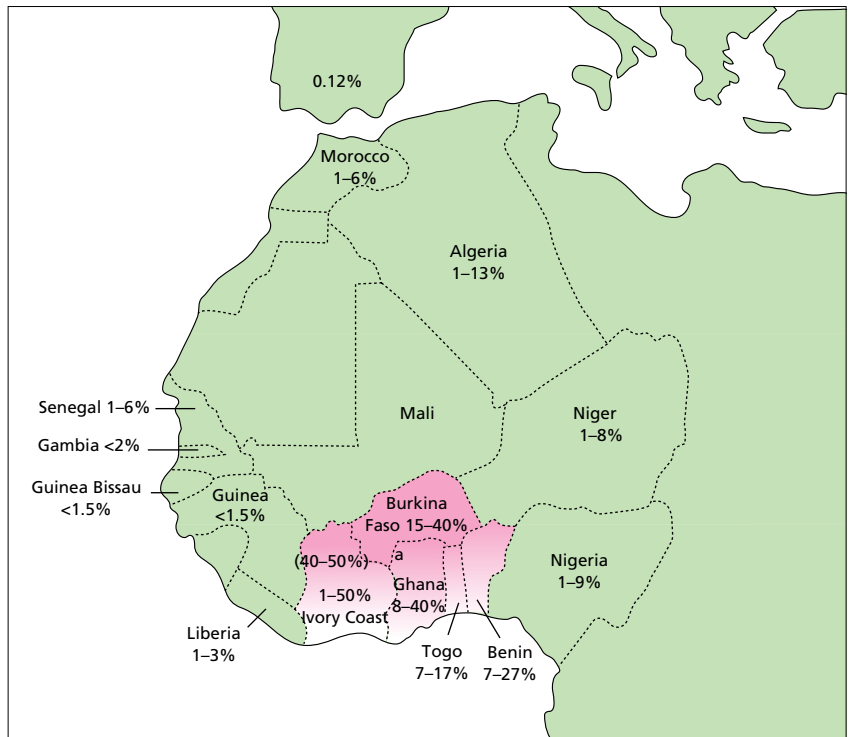


Fig. 5.1 Distribution of haemoglobin C in north-west Africa.

from Iraq, the identification being based on HPLC only [7].

There are also variant haemoglobins in which the haemoglobin C mutation is one of two mutations, specifically haemoglobin Arlington Park and haemoglobin C-Rothschild.

Haemoglobin C appears to protect from severe *falciparum* malaria [8] with homozygosity giving very high protection and heterozygosity giving moderate protection [9]. However, conflicting results have been reported from Mali where the incidence of malaria was found to be higher in people with haemoglobin C trait [10].

Oxyhaemoglobin C is prone to crystallise but crystals dissolve on deoxygenation so that obstruction of capillaries by cells containing crystals is not likely. In comparison with haemoglobin A, crystallisation is inhibited by haemoglobins F and A₂ [5].

Haemoglobin C trait

Haemoglobin C trait describes the heterozygous condition in which there is one normal β gene and one β^C gene. It is of no clinical significance but is of significance in counselling prospective parents. This is largely because of the possibility of sickle cell/haemoglobin C disease if one parent has haemoglobin C trait and the other has sickle cell trait.

Clinical features

There are usually no clinical features.

Laboratory features

Blood count

The haemoglobin concentration (Hb) is usually normal but microcytosis is common. There are conflicting data as to whether this results from coexisting α thalassaemia trait. In one study, individuals with C trait and with the normal complement of four α globin genes had a mean cell volume (MCV), on average, around the bottom of the normal range [11], whereas in another study there was no difference between C trait

and normal [12]. The mean cell haemoglobin concentration (MCHC) is, on average, higher than normal, usually around the top of the normal range, probably related to red cell dehydration. The red cell distribution width (RDW) is increased.

Blood film

The blood film (Fig. 5.2) is sometimes normal but usually shows microcytosis, target cells, irregularly contracted cells or a combination of these features.

Other investigations

Haemoglobin electrophoresis (Fig. 5.3), HPLC (Fig. 5.4) and capillary electrophoresis (Fig. 5.5) show haemoglobin A to constitute somewhat more than 50% of haemoglobin and haemoglobin C slightly less. The proportion of haemoglobin C is lower in those with coexisting α thalassaemia trait. In one study the mean percentage of haemoglobin C (plus A₂) was around 44% in those thought likely to have four α genes, around 37.5% in those thought likely to have three α genes and around 32% in those likely to have two α genes [13]. In another study the mean levels were 37% and 32% in those with four and three α genes, respectively [12]. In a single patient with haemoglobin C trait and haemoglobin H disease, the haemoglobin C was 24% [5]. In subjects with five α genes, the haemoglobin C percentage tends to be higher than in those with four α genes [14]. On cellulose acetate at alkaline pH, haemoglobin C has the same mobility as haemoglobins E, A₂ and O-Arab. On citrate agar or agarose gel at acid pH it can be separated from haemoglobins E and A₂ (same mobility as A) and from haemoglobin O-Arab (similar mobility to haemoglobin S). On HPLC, haemoglobin C can be separated from haemoglobin E and haemoglobin O-Arab but, depending on the specific instrument/reagent system, there may be overlap with haemoglobin A₂ and haemoglobin Lepore. Haemoglobin C can be detected immunologically, including in point-of-care testing devices [15].

Red cell density is increased, as a result of increased K—Cl cotransport, loss of intracellular potassium and resultant cellular dehydration [9]. Osmotic fragility is decreased. Red cell survival is normal or slightly reduced.

Diagnosis

Diagnosis is dependent on identifying haemoglobin A and haemoglobin C by at least two independent techniques, with haemoglobin C being present in a lower amount than haemoglobin A. DNA analysis using allele specific amplification or direct sequencing can be employed but is rarely necessary in routine clinical practice.

Haemoglobin C disease

Haemoglobin C disease describes the homozygous state in which there are two β^C

genes and no normal β gene. As a consequence, about 95% of total haemoglobin is haemoglobin C with the remainder being haemoglobins A₂ and F. Homozygosity for haemoglobin C leads to a clinically mild, chronic haemolytic anaemia.

Clinical features

Individuals with haemoglobin C disease either have a normal Hb or are mildly or moderately anaemic. Because of the chronic haemolysis, there is an increased incidence of gallstones. The spleen may be enlarged.

Laboratory features

Blood count

The Hb ranges from about 80 g/l up to normal. There is often marked microcytosis. In one study, patients with a normal complement of

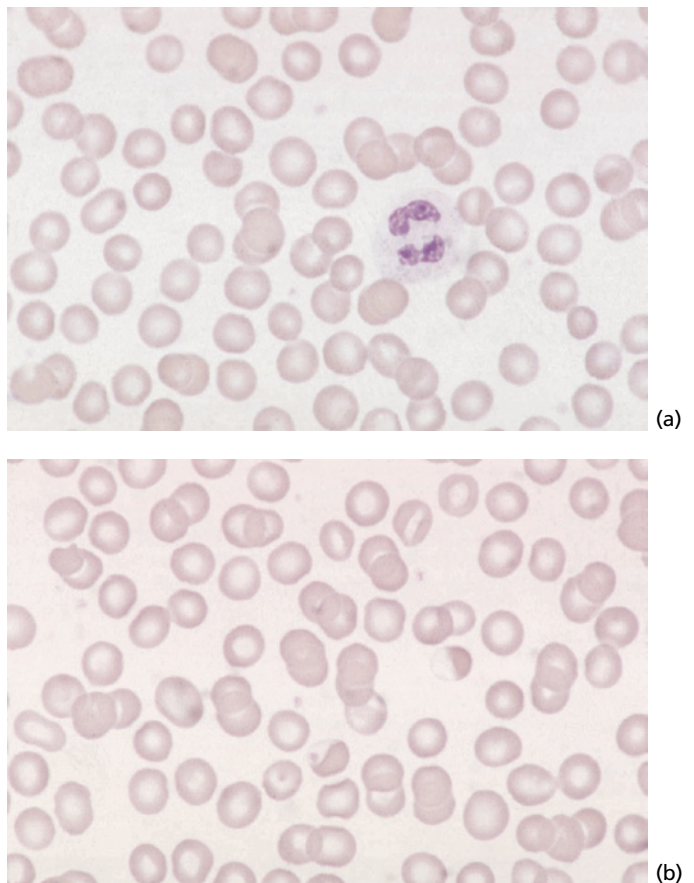


Fig. 5.2 Blood films from four patients with haemoglobin C trait showing the range of features that may be observed: (a) normal film; (b) one irregularly contracted cell and one hemi-ghost; (*Continued on p. 304.*)

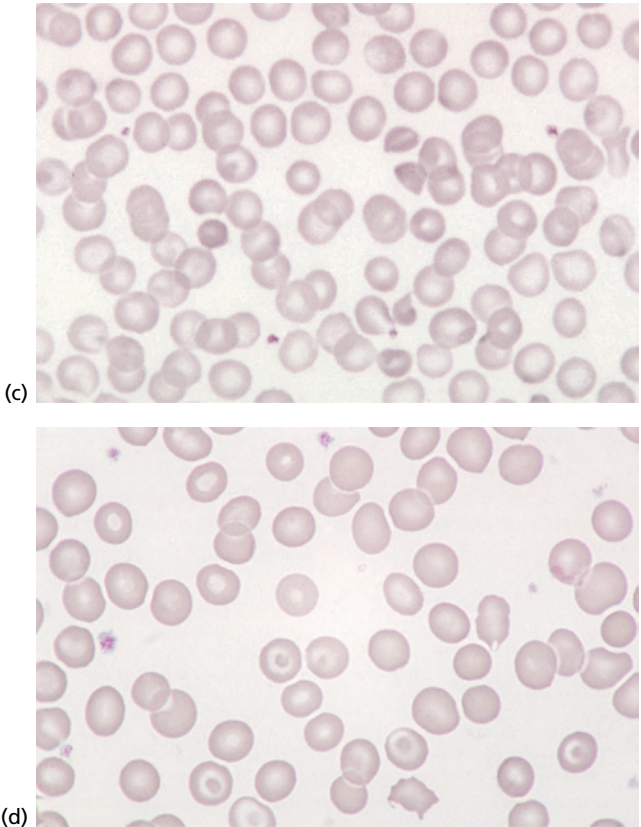


Fig. 5.2 *Continued.* (c) irregularly contracted cells; and (d) target cells and other poikilocytes. May-Grünwald-Giemsa (MGG) $\times 100$ objective.

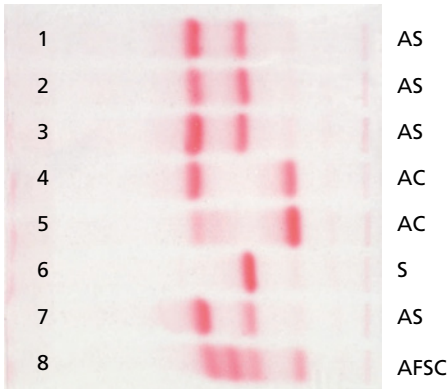


Fig. 5.3 Haemoglobin electrophoresis on cellulose acetate at alkaline pH showing haemoglobin C trait (lane 4) and haemoglobin C/ β^+ thalassaemia compound heterozygosity (lane 5).

four α genes and no iron deficiency had, on average, an MCV of 55 fl [11]. The MCHC is, on average, around the top of the normal range

and the proportion of hyperdense cells is increased. The cause of the microcytosis and increased MCHC is activation of the K-Cl cotransporter, which leads to loss of water from the cell; cells are therefore smaller, denser and less deformable than normal [5]. The reticulocyte count is mildly elevated, usually 2–4%. Thrombocytosis is common in children [16].

Blood film

The blood film (Fig. 5.6) characteristically shows numerous target cells and numerous irregularly contracted cells [17]. There is also microcytosis. There may be occasional nucleated red blood cells (NRBC). Occasional cells may contain haemoglobin C crystals (Figs 5.6c and 5.7). Crystals may be tetragonal or hexagonal. Usually all the haemoglobin in a cell has been incorporated into the crystal so that the crystal is in a cell that otherwise appears empty of haemoglobin. The

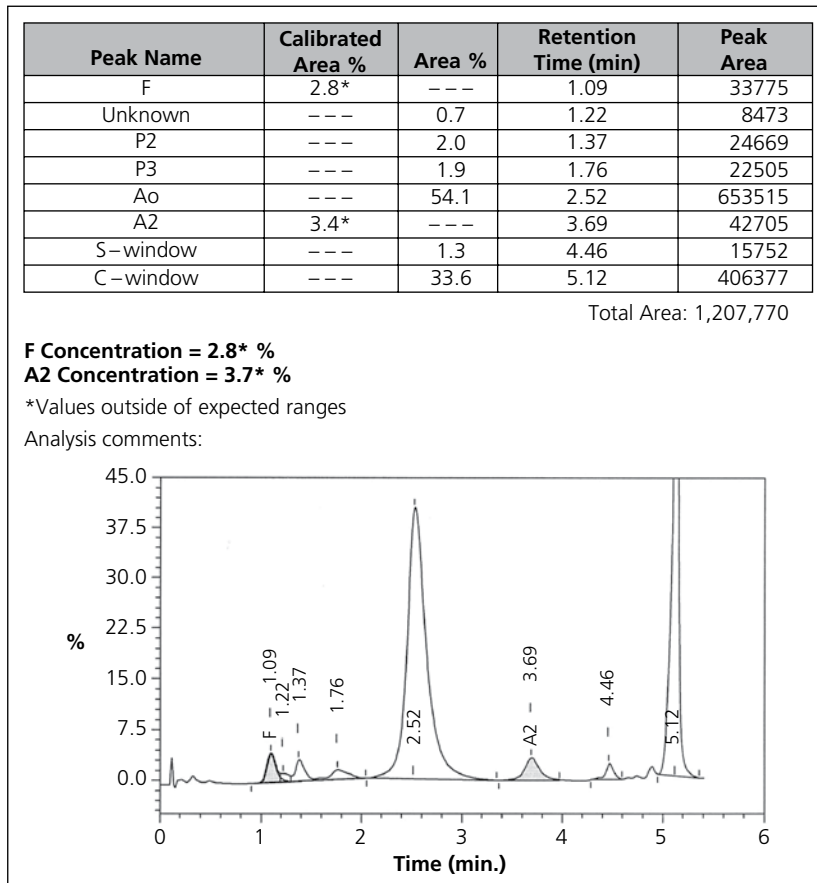


Fig. 5.4 High performance liquid chromatography (HPLC) chromatogram (Bio-Rad Variant II) in haemoglobin C trait. Peaks from left to right are haemoglobin F (shaded), two small peaks representing post-translationally modified haemoglobin A, haemoglobin A₀, haemoglobin A₂ (shaded), two small peaks representing post-translationally modified haemoglobin C and haemoglobin C₀.

majority of crystals are 6–10 µm in length and 2–3 µm in diameter with pointed ends. Crystals can be present *in vivo* but can also form *in vitro*, particularly if the film dries slowly [18].

Both crystals and NRBC are more often seen in patients who have been splenectomised.

Other investigations

Haemoglobin electrophoresis (Fig. 5.8) and HPLC (Fig. 5.9) show that haemoglobin C comprises almost all the haemoglobin. Haemoglobin F can be slightly elevated but does not usually exceed 3%.

The percentage of dense cells is markedly increased. Osmotic fragility is markedly reduced. Bilirubin concentration is normal or

increased. Oxygen affinity is reduced as a result of reduction of intracellular pH, rather than any alteration of the oxygen affinity of haemoglobin C [5]. Red cell survival is reduced to about a third of normal [5].

The bone marrow shows erythroid hyperplasia and characteristic dyserythropoietic features with an irregular nuclear membrane (Fig 5.10). On ultrastructural examination, there can also be duplication of the nuclear membrane (Fig 5.11).

Diagnosis

Diagnosis is dependent on identifying haemoglobin C as the sole variant haemoglobin, in the absence of haemoglobin A, by at least two

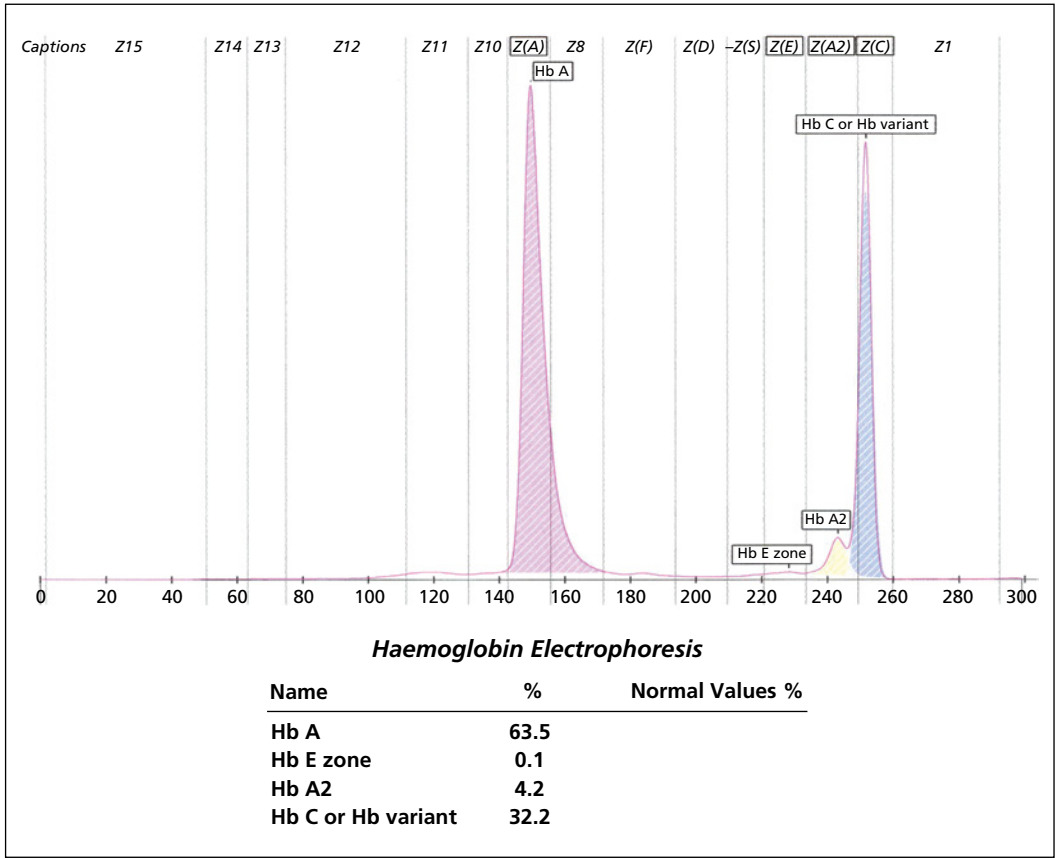


Fig. 5.5 Capillary electrophoresis (Sebia Capillarys 3) in haemoglobin C trait showing haemoglobins A, A₂ and C. Note that haemoglobins A₂ and C overlap.

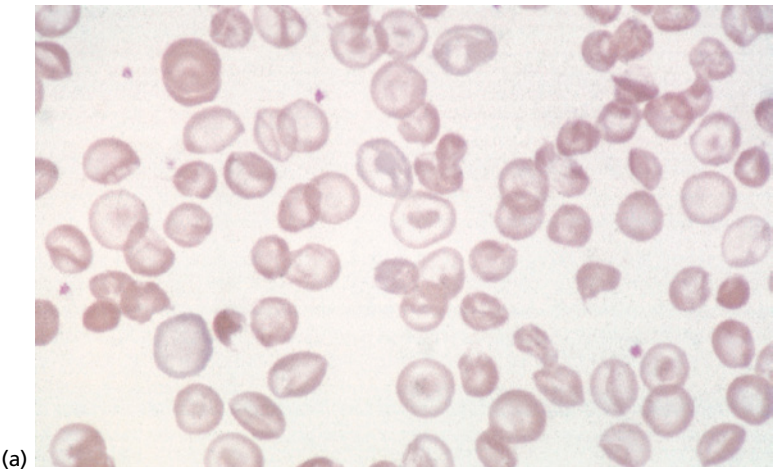
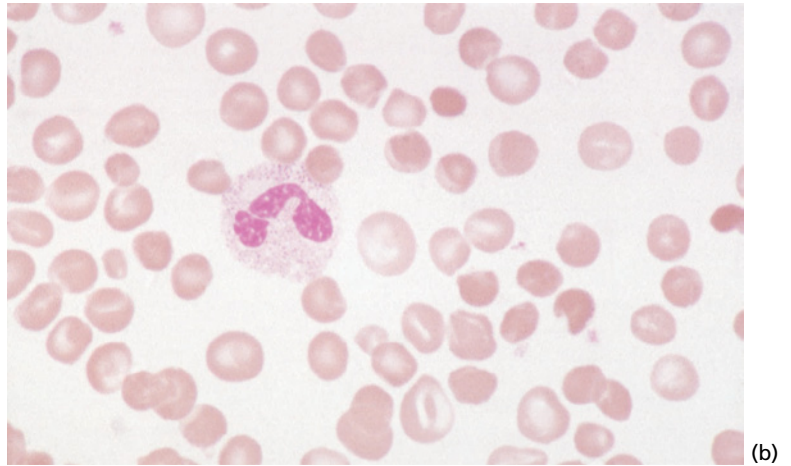
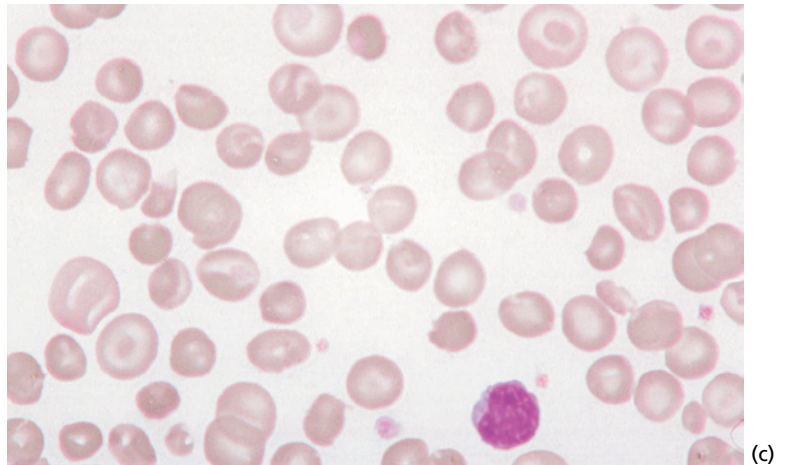


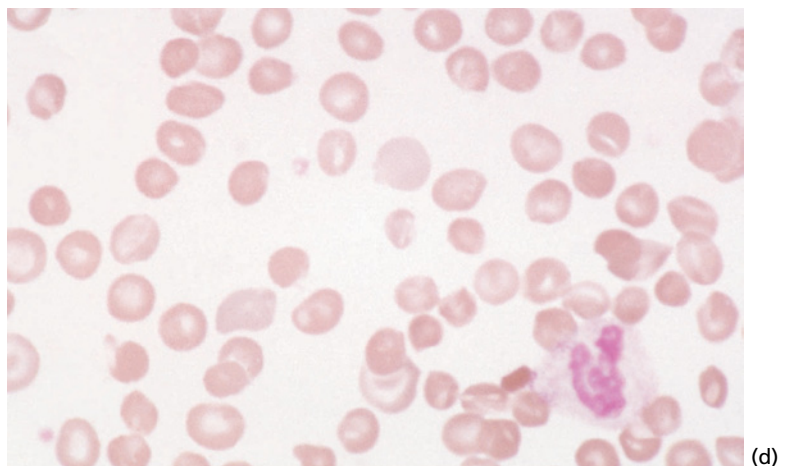
Fig. 5.6 Blood films from four patients with haemoglobin C disease showing the range of features that may be observed: (a) target cells and irregularly contracted red cells;



(b)



(c)



(d)

Fig. 5.6 *Continued.*

(b) irregularly contracted cells and only an occasional target cell; (c) target cells and irregularly contracted cells; and (d) irregularly contracted cells and one haemoglobin C crystal (adjacent to the neutrophil). MGG $\times 100$.

independent techniques. The differential diagnosis includes compound heterozygosity for haemoglobin C and β^0 thalassaemia and, if cellulose acetate electrophoresis is the primary method used, compound heterozygosity for haemoglobin C and either haemoglobin E or haemoglobin C-Harlem. The latter two compound heterozygous states are rare. Unless the MCV and mean cell haemoglobin (MCH) are normal, DNA analysis or family studies are needed to distinguish between homozygous haemoglobin C and haemoglobin C/ β^0 thalassaemia.

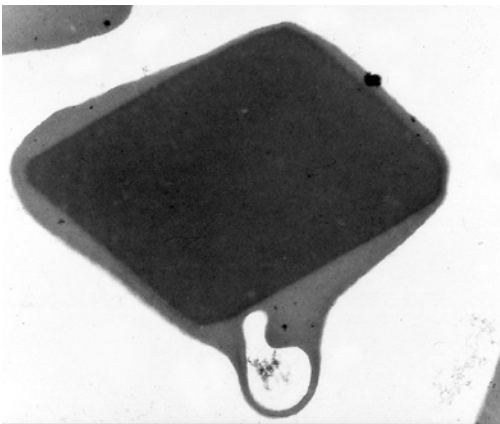


Fig. 5.7 Ultrastructural examination of an erythrocyte containing a haemoglobin C crystal. (Courtesy of the late Professor Sunitha N. Wickramasinghe.)

Haemoglobin C/ β thalassaemia

Haemoglobin C may be coinherited with either β^0 or β^+ thalassaemia. The latter is more common because β^+ thalassaemia is more common than β^0 in the ethnic groups that are most likely to inherit haemoglobin C. This compound heterozygous state is observed particularly in those with African ancestry but has also been reported in Italians and Turks.

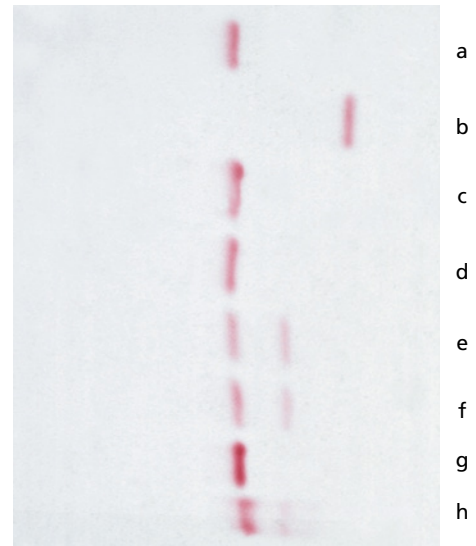


Fig. 5.8 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in haemoglobin C disease (lane b); other lanes show either haemoglobin A alone or haemoglobin A plus haemoglobin S.

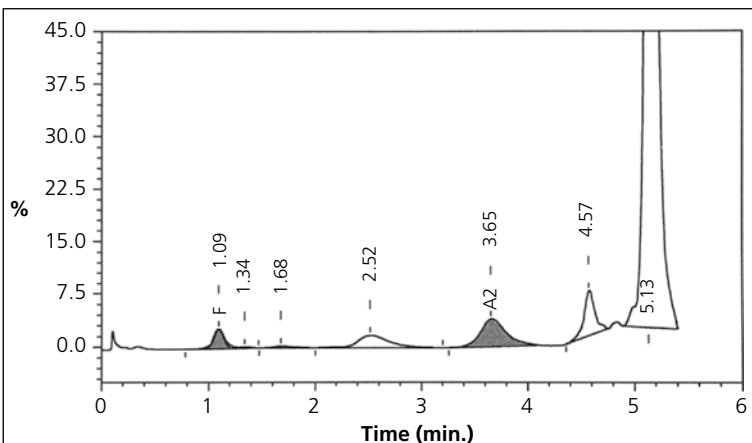


Fig. 5.9 HPLC chromatogram (Bio-Rad Variant II) in haemoglobin C homozygosity; peaks, from left to right, are haemoglobin F (shaded), unidentified, haemoglobin A₂ (shaded), glycosylated haemoglobin C and haemoglobin C₀; the shoulder on the left of the haemoglobin C₀ peak represents post-translationally modified haemoglobin C.

Fig. 5.10 Bone marrow aspirate from a patient with haemoglobin C disease showing erythroid hyperplasia and an irregular nuclear margin. MGG $\times 100$.

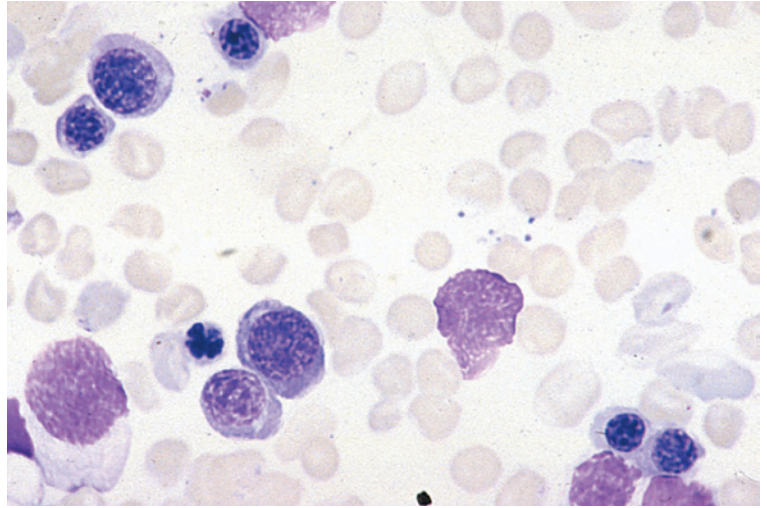
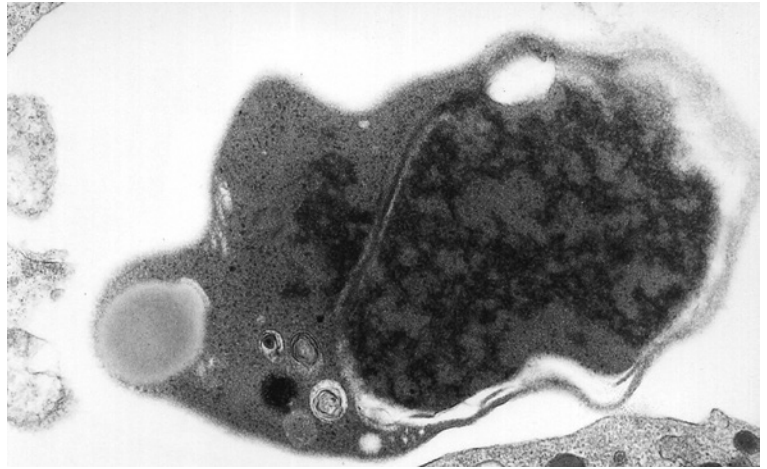


Fig. 5.11 Ultrastructural examination showing the characteristic abnormality of the nuclear membrane in homozygosity for haemoglobin C. (Courtesy of the late Professor Sunitha N. Wickramasinghe.)



Clinical features

Compound heterozygosity for haemoglobin C and β thalassaemia leads to an often asymptomatic condition but there can be moderately severe anaemia and splenomegaly. If haemoglobin C is coinherited with β^0 thalassaemia or severe β^+ thalassaemia, the clinical picture resembles mild thalassaemia intermedia with moderately severe anaemia, splenomegaly and sometimes hypersplenism. Worsening anaemia consequent on parvovirus B19 infection has been observed. If haemoglobin C is coinherited with mild β^+ thalassaemia, the features are similar to those of homozygosity for haemoglobin C. There is a mild to moderate

haemolytic anaemia and some splenomegaly. Fatal spontaneous rupture of the spleen during pregnancy has been reported in a patient with previously undiagnosed haemoglobin C/ β thalassaemia; the spleen showed extramedullary haemopoiesis [19].

Laboratory features

Blood count

The Hb varies from 70 to 100 g/l in haemoglobin C/ β^0 thalassaemia. In haemoglobin C/ β^+ the Hb can be reduced or, occasionally, normal. The MCV is markedly reduced. The reticulocyte count is moderately elevated.

Blood film

The blood film (Fig. 5.12) shows hypochromia, microcytosis, target cells and irregularly contracted cells. There is more anisocytosis and poikilocytosis than in haemoglobin C disease, particularly in cases of haemoglobin C/ β^0 thalassaemia. Haemoglobin C crystals are sometimes present.

Other investigations

The major haemoglobin is haemoglobin C with haemoglobin F usually being 2–10% (most often greater than 5%). Haemoglobin A can be totally absent (haemoglobin C/ β^0 thalassaemia) or,

when haemoglobin C is coinherited with a mild β^+ thalassaemia, up to 20–30% of total haemoglobin (Figs 5.13 and 5.14).

Osmotic fragility is markedly reduced.

Diagnosis

Diagnosis of haemoglobin C/ β^+ thalassaemia is straightforward, being dependent on identification of haemoglobin A and haemoglobin C by two independent techniques with haemoglobin C being more than haemoglobin A. Diagnosis of haemoglobin C/ β^0 thalassaemia can be more problematic since it is not infrequent for patients with haemoglobin C homozygosity to have

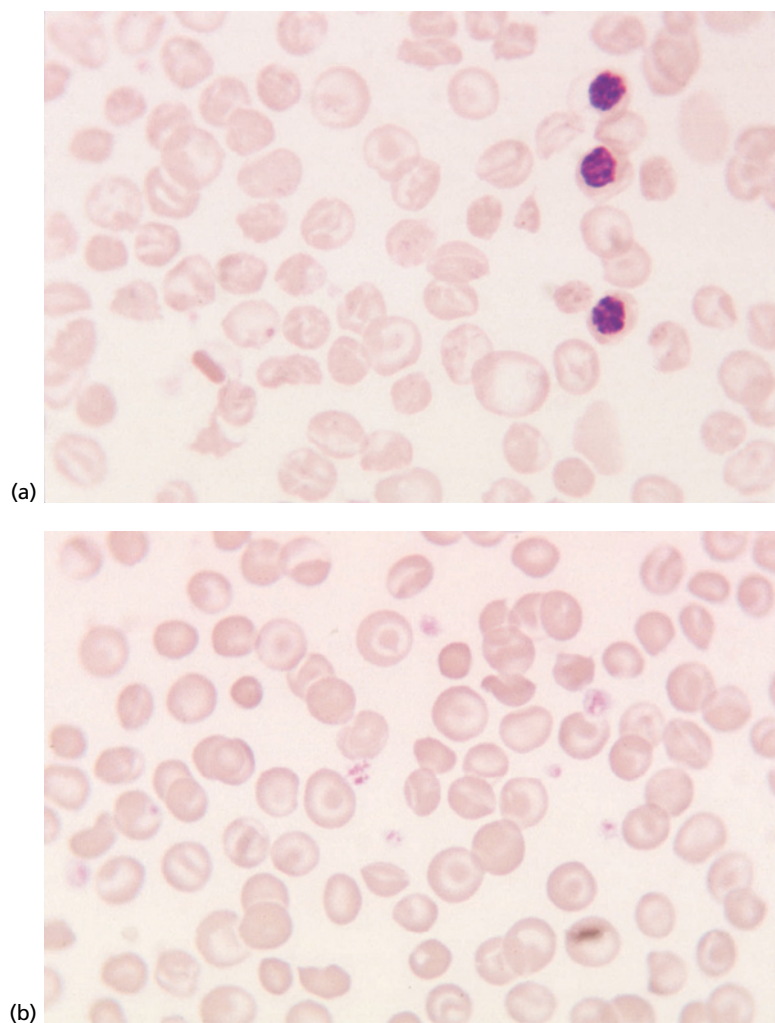


Fig. 5.12 Blood films in two patients with haemoglobin C/ β thalassaemia compound heterozygosity: (a) haemoglobin C/ β^0 thalassaemia showing hypochromia, irregularly contracted cells, three nucleated red blood cells and a cell containing a haemoglobin C crystal; (b) haemoglobin C/ β^+ thalassaemia showing irregularly contracted cells and target cells; the electrophoretic pattern of this patient is shown in Fig. 5.3 (lane 5). MGG $\times 100$.

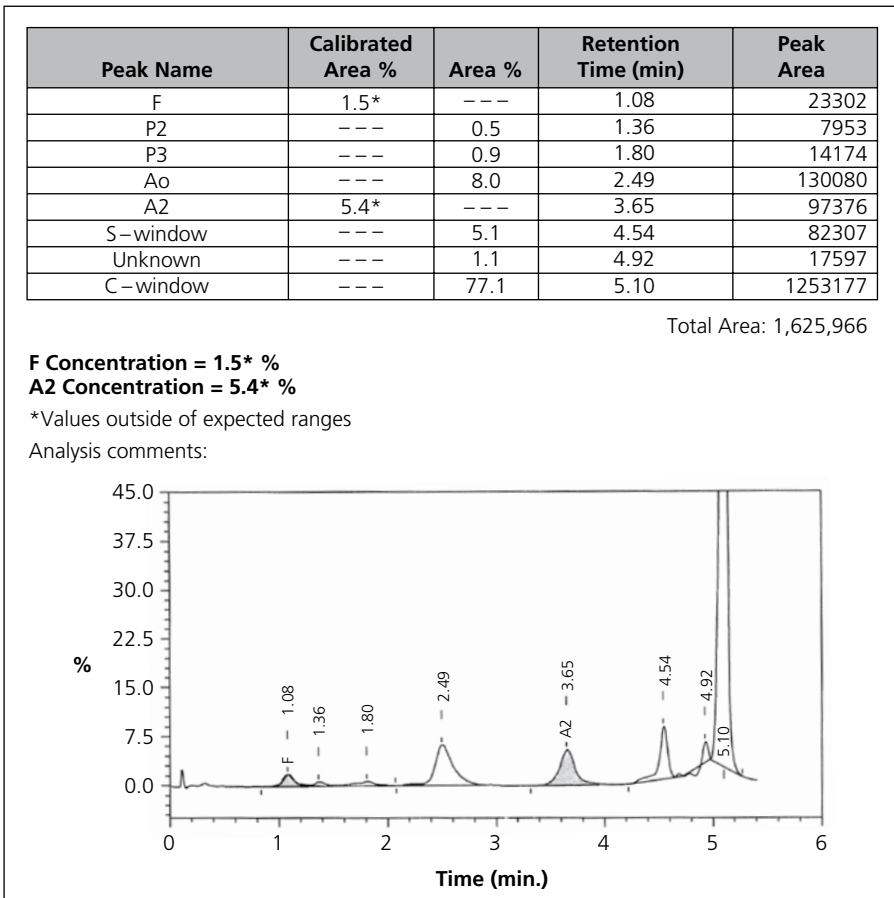


Fig. 5.13 HPLC chromatogram (Bio-Rad Variant II) of a patient with haemoglobin C/ β^+ thalassaemia. The peaks from left to right are: haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobin A₀, haemoglobin A₂ (shaded), post-translationally modified haemoglobin C (two peaks) and haemoglobin C₀. The red cell indices were red cell count (RBC) $6.8 \times 10^{12}/l$, haemoglobin concentration (Hb) 128 g/l, mean cell volume (MCV) 55.4 fl, mean cell haemoglobin (MCH) 18.7 pg and red cell distribution width (RDW) 19.

microcytosis, as a result of cellular dehydration or coexisting α thalassaemia trait. A distinction can be made by family studies or deoxyribonucleic acid (DNA) analysis.

Coinheritance of haemoglobin C and other variant haemoglobins or thalassaemias

Coinheritance of haemoglobin C and either haemoglobin Lepore or $\delta\beta$ thalassaemia leads to a clinically mild disease resembling coinheritance of haemoglobin C and mild β^+

thalassaemia. In haemoglobin C/haemoglobin Lepore compound heterozygosity, haemoglobin C is around 80%, haemoglobin Lepore 10–15% and haemoglobin F 6–12%. Haemoglobin Lepore-Boston inhibits the crystallisation of haemoglobin C [5]. In haemoglobin C/ $\delta\beta$ thalassaemia, haemoglobin C is around 75% and haemoglobin F around 25% of total haemoglobin. Coinheritance of haemoglobin C and deletional hereditary persistence of fetal haemoglobin is also clinically mild.

Haemoglobin C crystallisation is accelerated in haemoglobin C heterozygotes who are also

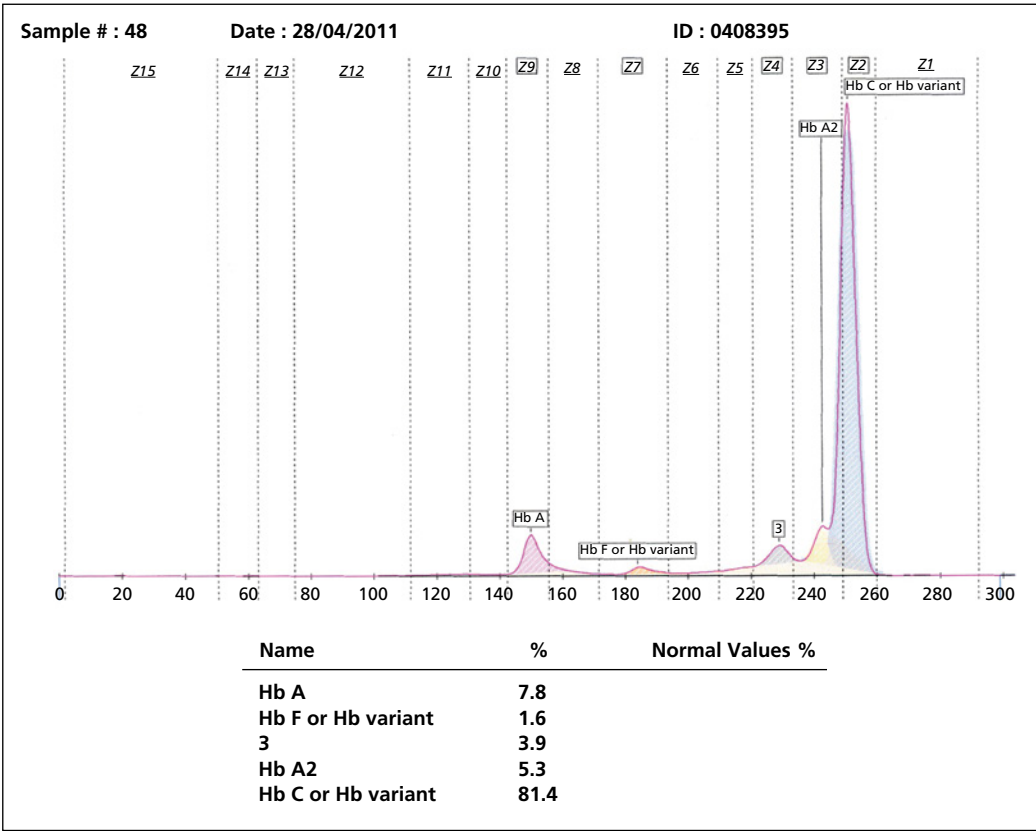


Fig. 5.14 Capillary electrophoresis (Sebia Capillars 2) of a patient with haemoglobin C/ β^+ thalassaemia. Note that the A₂ peak overlaps with the haemoglobin C peak. Same patient as Fig. 5.13.

heterozygous for haemoglobin Korle-Bu, leading to a mild haemolytic anaemia with microcytosis and increased numbers of hyperdense cells [20, 21]. Crystals are cubic [20]. Compound heterozygosity for C and N-Baltimore also leads to accelerated crystallisation of haemoglobin C in comparison with haemoglobin C trait [21]. The phenotype may be intermediate in severity between that of heterozygosity and homozygosity for haemoglobin C. If haemoglobin Riyadh is coinherited with haemoglobin C, crystallisation is retarded and microcytosis is usually the only feature [21, 22]. Haemoglobin C coinherited with haemoglobin K-Woolwich or haemoglobin P-Galveston does not differ in severity from haemoglobin C trait [23].

Coinheritance of haemoglobins C and E has been described [6, 24–27]. It has been noted that the haemoglobin E percentage tends to be

higher than in haemoglobin E trait [6, 25]. For example, three children had haemoglobin E plus A₂ of 33–37% (cf. 25–30% in haemoglobin E simple heterozygotes), haemoglobin C of 54–56% and haemoglobin F of 2.1–5.8% [6]. In one patient, who was clinically well, the haematocrit and reticulocyte count were normal [24]; the red cells were normocytic and normochromic but showed cytoplasmic folding and stomatocyte formation. In a second patient there was a mild anaemia (Hb 99 g/l) with red cell indices suggestive of thalassaemia trait; haemoglobin C was 60% and haemoglobin E 39% [25]. An adult patient had a normal Hb, red cell indices suggesting thalassaemia trait, a blood film showing target cells and irregularly contracted cells and HPLC showing 38% E plus A₂ and 60% haemoglobin C [26]. Two of the three children mentioned above were

anaemic (Hb 75 and 95 g/l) and red cells were markedly microcytic (MCV 52 and 61 fl) in the absence of coexisting α thalassaemia trait (although iron deficiency was not excluded) [6]. Figure 5.15 shows the blood film and other investigations of an eight-month-old baby with haemoglobin C/haemoglobin E compound heterozygosity [27].

Coinheritance of haemoglobin C and the α chain variant, haemoglobin G-Philadelphia, does not differ clinically or haematologically from haemoglobin C trait although haemoglobin C crystallisation is accelerated [5]. Electrophoresis on cellulose acetate at alkaline pH shows four bands, haemoglobin A, haemoglobin G, haemoglobin C and a slow-moving haemoglobin G-C hybrid haemoglobin (Fig. 5.16). On agarose gel at acid pH there are only two bands: A plus G-Philadelphia and C plus C-G hybrid (Fig. 5.17).

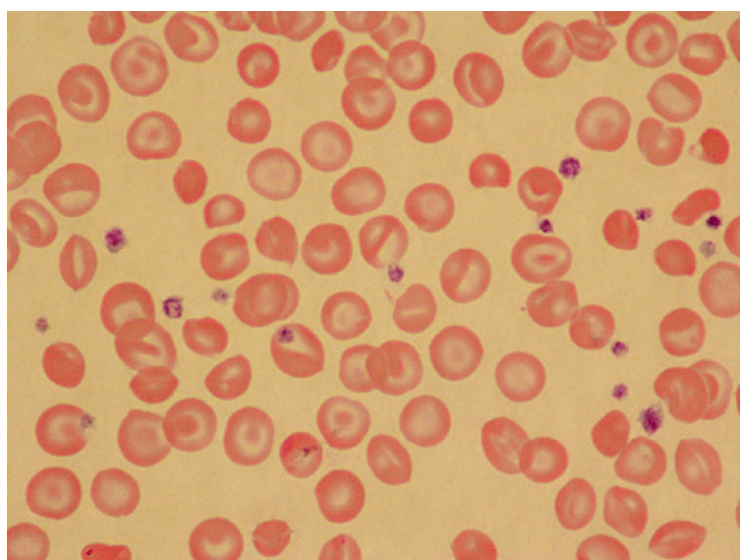
Coexistence of haemoglobin C heterozygosity and the genotype of haemoglobin H disease leads to an atypical form of haemoglobin H disease. One reported case had a chronic haemolytic anaemia and significant splenomegaly [28]. There was marked microcytosis. Only haemoglobins A and C (20%) were detected on haemoglobin electrophoresis and only occasional inclusion-containing cells were found on a haemoglobin H preparation.

As is the case with the sickle cell mutation, the haemoglobin C mutation can occur as one of two mutations on a chromosome. Haemoglobin Arlington Park is $\alpha_1\beta_2^{7\text{ Glu} \rightarrow \text{Lys}, 96\text{ Lys} \rightarrow \text{Glu}}$. There is no net change in charge relative to haemoglobin A so that this variant haemoglobin is electrophoretically silent. Nevertheless, it interacts in the same manner as haemoglobin C with haemoglobin S to give a clinically significant sickling disorder. Haemoglobin C-Rothschild has been described in a heterozygote; it moves cathodal to haemoglobin C at alkaline pH and between haemoglobins S and C on citrate agar at acid pH.

Haemoglobin E

Haemoglobin E is a β chain variant, $\alpha_1\beta_2^{27\text{ Glu} \rightarrow \text{Lys}}$, which is common in South-East Asia (Table 5.2) [29–38]. It is one of the two most common variant haemoglobins in the world, together with haemoglobin S. It was first described by Chernoff and colleagues in 1954 [39] and independently in the same year by Itano and colleagues [40]. The highest prevalence is in some parts of Thailand, Cambodia and Laos. Thailand and Myanmar (previously Burma) have an overall prevalence of around 14–15%. Allele frequency in Thailand varies from 8% to 50–70%, being highest in north-eastern Thailand. Haemoglobin E is also found in Sri Lanka,

Fig. 5.15 Investigations of an eight-month-old baby with compound heterozygosity for haemoglobins C and E. (a) Blood film showing target cells and irregularly contracted cells (MGG \times 100); the red cell indices were RBC $4.45 \times 10^9/\text{l}$, Hb 92 g/l, MCV 57.8 fl, MCH 20.7 pg and MCHC 358 g/l; (Continued on pp. 314–315.)



(a)

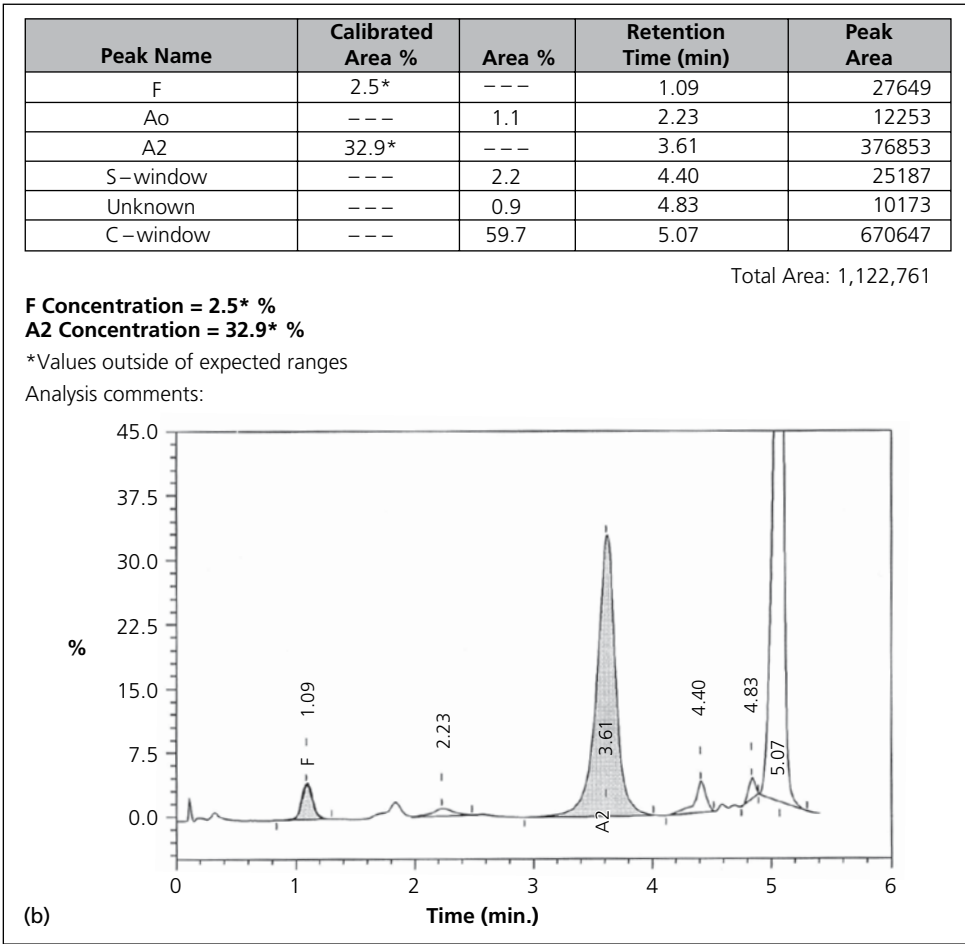


Fig. 5.15 *Continued.* (b) HPLC chromatogram (Bio-Rad variant II) showing, from left to right, haemoglobin F, two small peaks of post-translationally modified haemoglobin E, haemoglobins E₀ plus A₂ (32.9%), two small peaks of modified haemoglobin C and haemoglobin C₀ (59.7%);

north-eastern India (Bengal and Assam), Bangladesh, Pakistan, Nepal, Vietnam, Malaysia, the Philippines, Indonesia and Turkey. Although haemoglobin E is prevalent in Sri Lanka, it is not prevalent in southern India; it is thought to have reached Sri Lanka during migration from north-eastern India during the fifth century BCE. Occasional cases have been observed in individuals of apparent northern European Caucasian descent and a single affected family has been observed in former Czechoslovakia. There are also variant haemoglobins in which the haemoglobin E mutation is one of two mutations, specifically haemoglobin Corbeil and haemoglobin T-Cambodia.

The β^E chain is synthesised at a reduced rate in comparison with β^A . This is because the mutation both leads to slower excision of intervening sequence 1 and also creates a false splicing site towards the 3' end of exon 1 so that there is a proportion of abnormally spliced messenger ribonucleic acid (mRNA). Post-transcriptional processing of the latter is abnormal. The result of the reduced rate of synthesis of β^E chain, and therefore of haemoglobin E, is that heterozygotes, compound heterozygotes and homozygotes have mild β thalassaemia features, with microcytic hypochromia. Haemoglobin E is therefore a thalassaemic haemoglobinopathy.

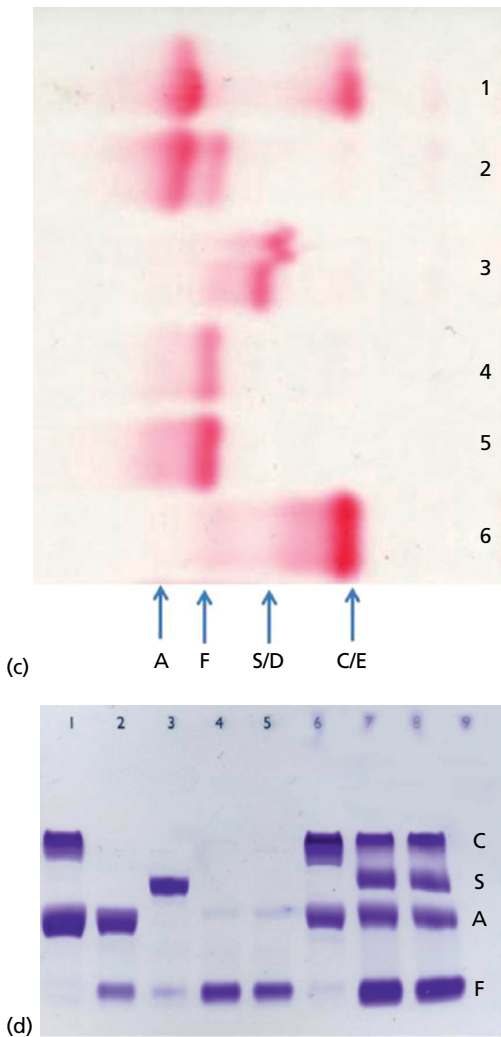


Fig. 5.15 *Continued.* (c) cellulose acetate electrophoresis at alkaline pH, showing the patient in lane 6; (d) acid agarose electrophoresis showing the patient in lane 6 (haemoglobin E has the same mobility as haemoglobin A at this pH).

The α :non- α chain synthesis ratio is 1.2–2.1 in heterozygotes [41]. Haemoglobin E also has weakened $\alpha_1\beta_1$ contacts, leading to instability in conditions of increased oxidant stress. The most significant clinical consequences occur if haemoglobin E is coinherited with β thalassaemia trait, often leading to thalassaemia major or intermedia. Homozygosity for haemoglobin E produces a clinically mild condition and is thus of much less significance.

Hb electrophoresis

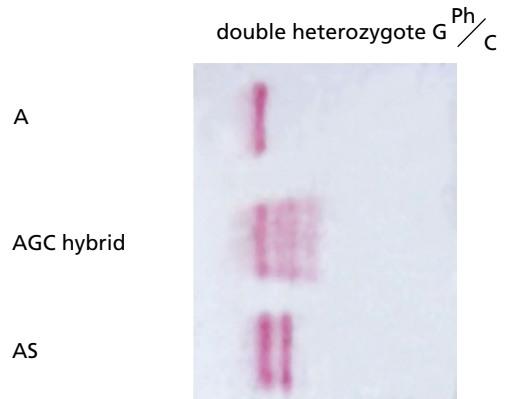


Fig. 5.16 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in a patient with heterozygosity for both haemoglobin C and the α -chain variant, haemoglobin G-Philadelphia; four bands are apparent representing, from left to right, haemoglobin A, haemoglobin G, haemoglobin C and hybrid C/G.

Haemoglobin E heterozygosity may protect from malaria, although this is uncertain [38, 42], and may protect from severe *falciparum* malaria [43]. Haemoglobin E homozygosity is protective against malaria [42].

Haemoglobin E trait

Haemoglobin E trait is an asymptomatic condition with no clinical significance except for the possibility of homozygous or compound heterozygous states in the children of heterozygotes.

Clinical features

There are usually no clinical features although increased susceptibility to oxidant-induced haemolysis has been suspected.

Laboratory features

Blood count

Some patients have a normal blood count. Others have an increased red cell count (RBC) and reduced MCH and MCV, with or without mild anaemia. The Hb does not usually fall much below 120 g/l. The MCHC is normal or, occasionally, increased. The red cell indices often resemble

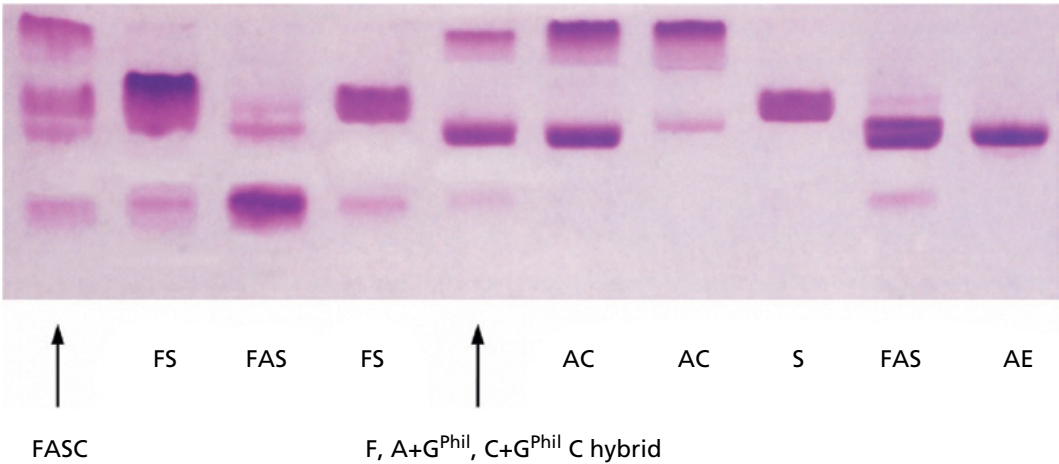


Fig. 5.17 Haemoglobin electrophoresis on agarose gel at acid pH in a patient with heterozygosity for both haemoglobin C and the α -chain variant, haemoglobin G-Philadelphia; at this pH, since haemoglobin G moves with haemoglobin A and the hybrid haemoglobin moves with haemoglobin C, only two bands are apparent (apart from a faint haemoglobin F band); FASC indicates a control sample containing haemoglobins F, A, S and C.

Table 5.2 Prevalence of haemoglobin E carriers in various countries.

Country	Percentage
India	0–3.5*
Pakistan	0.5–1
Bangladesh	4
Bhutan	1.5–6.5
Nepal	<1
Sri Lanka	0–13† (overall 0.5%) and 0–3.3% in different districts reported
Myanmar	1–33
Thailand	8–40‡
Laos	20–40‡
Cambodia	15–30‡
Vietnam	2–4§
Southern China	1–2.5
Malaysia	1–40¶
Indonesia	1–13
Philippines	≈ 1
Turkey	0.5–1
Iran	0.02
Jamaica	0.007

* But 22% in Kolkata, 60% in some parts of west Bengal and 50–80% in Assam.
† Common in the Veddah; equally common in Tamils and Sinhalese [38].
‡ Haemoglobin E occurs in 70% of the So people of north-east Thailand, in 50% of the Khmer people on the borders of Laos, Thailand and Cambodia and in 50% of the Kachari people in Assam [34].
§ Much higher incidence among Khmer population in Vietnam (20–30%) and in certain other ethnic groups including the Ede and the Vân Kiê (5–50%); ranges from zero to 36% in eight ethnic groups in southern Vietnam [37].
¶ More frequent in the aboriginal population than in the Malays [31].
From references [29–38] and other sources.

those of thalassaemia trait [44]. It is not uncommon for individuals with haemoglobin E trait to also have a deletion of one or two α genes. However, even those with a full complement of α genes can have microcytosis and mild anaemia. In a report of 34 such cases the average Hb was 124 g/l, the MCV 79.7 fl and the MCH 26.2 pg [45].

Blood film

The blood film (Fig. 5.18) may be normal or may show hypochromia, microcytosis, target cells, irregularly contracted cells, basophilic stippling or any combination of these features.

Other investigations

Haemoglobin electrophoresis at alkaline pH (Fig. 5.19) shows the variant haemoglobin to have the same mobility as haemoglobins C and A₂. On citrate agar or agarose gel at acid pH the mobility of haemoglobin E is the same as that of haemoglobin A and A₂. Haemoglobin E has a characteristic mobility on isoelectric focusing, being well separated from haemoglobin A and moving close to haemoglobins C and A₂. On HPLC it is easily separated from haemoglobins A and C but with some instruments coelutes with haemoglobin A₂ (Fig. 5.20).

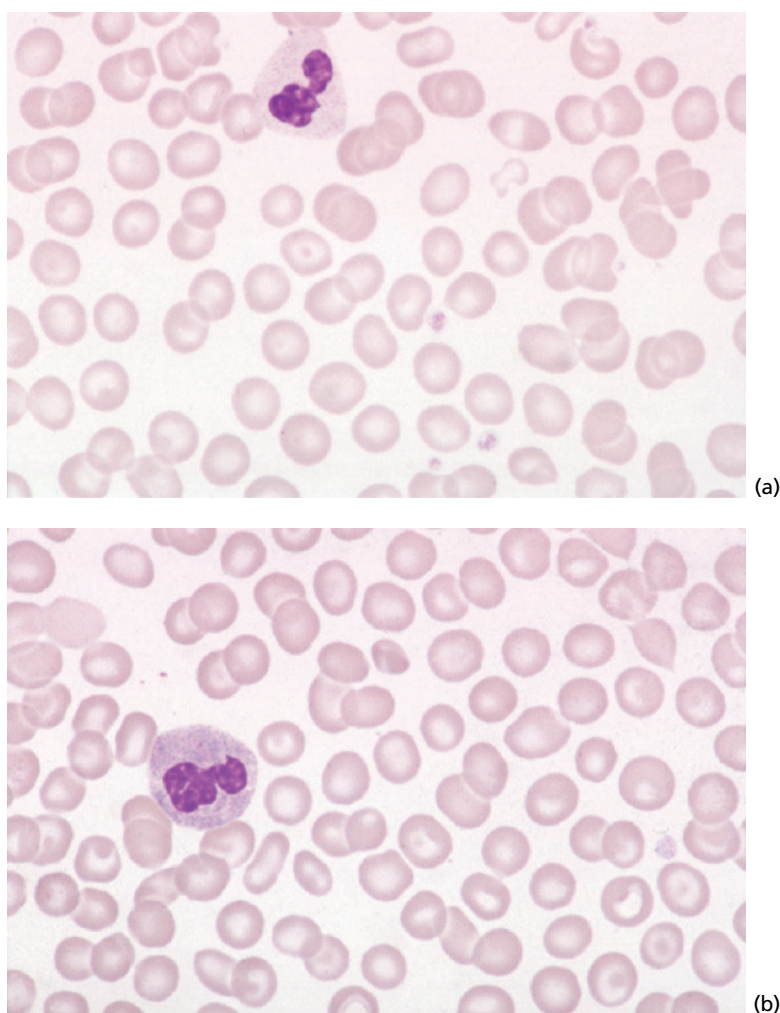


Fig. 5.18 Blood film in two patients with haemoglobin E trait showing: (a) microcytosis, minimal hypochromia and one target cell and (b) microcytosis (the MCV was 72 fl) and target cells. MGG $\times 100$.

Haemoglobin E can be separated from haemoglobin A₂ on capillary electrophoresis, permitting the latter to be measured (Fig. 5.21). The haemoglobin A₂ percentage is then often found to be increased in patients with haemoglobin E trait [46–49]; in one study there was a mean value of 3.4% in comparison with a mean normal of 2.6% [48] and in a second study the mean value was 4.0%, with six of seven results being elevated [49]. Haemoglobin E can also be detected by immunoassay [15].

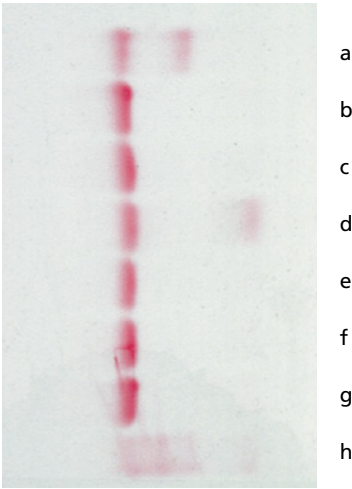


Fig. 5.19 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in a patient with haemoglobin E trait (lane d) note that the variant haemoglobin plus haemoglobin A₂ comprises only about 30% of total haemoglobin.

In haemoglobin E heterozygotes, the variant usually comprises 30% or less of total haemoglobin. Above 39% haemoglobin E suggests that the diagnosis is E/ β thalassaemia, not E trait [41]. A trimodal distribution of the variant haemoglobin is found, with modal percentages of 29.1%, 27.3% and 17.4%, correlating with the presence of four, three or two α globin genes [45]. Individuals with less than 25% haemoglobin E almost always have coexisting α thalassaemia trait [45]. When an individual is heterozygous for haemoglobin E and also has the genotype of haemoglobin H disease, the percentage of haemoglobin E is even lower, sometimes as low as 10%. On the basis of extensive experience in Thailand, Fucharoen has further reported that heterozygotes have 25–30% haemoglobin E when three or four α genes are present, 19–21% when two α genes are present and 13–15% when the genotype is that of haemoglobin H disease [41]. A single patient has been reported with a β^{++} thalassaemia mutation in *cis* to the haemoglobin E mutation leading to a haemoglobin E percentage of only 3.1% [50]. The percentage of haemoglobin E can also be lowered by coexisting iron deficiency. It should be noted that the percentages of haemoglobin E reported in various haemoglobin E syndromes are likely to represent haemoglobin E plus haemoglobin A₂.

Haemoglobin F can be slightly increased, being more than 1% (up to 5.7%) in 20 of 74 Thai patients [51]; this was mainly attributable to coinheritance of a polymorphism in the *BCL11A*

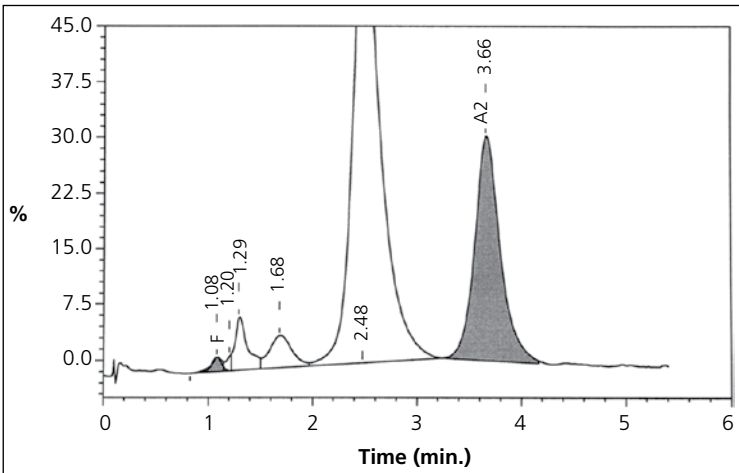
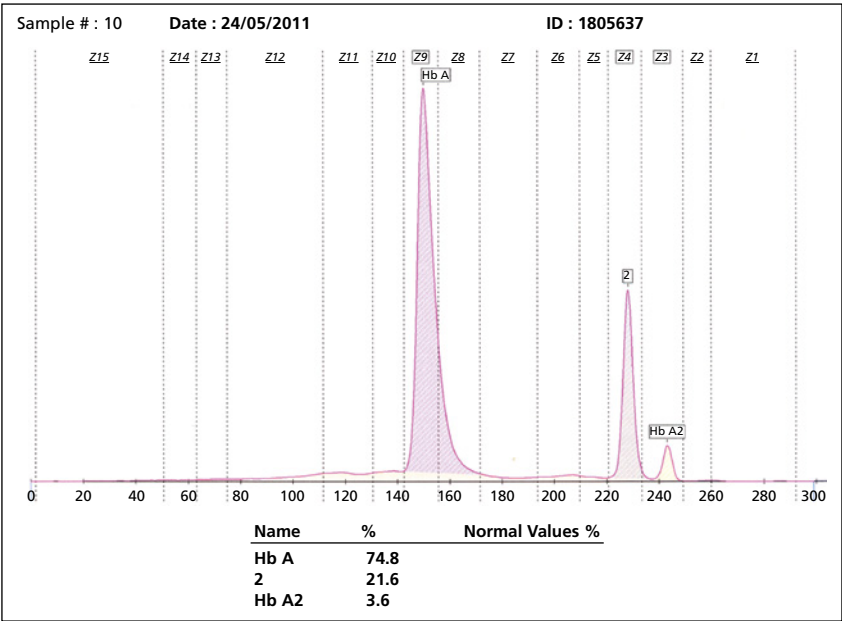


Fig. 5.20 HPLC chromatogram (Bio-Rad Variant II) from a patient with haemoglobin E heterozygosity; haemoglobin E appears in the A₂ window with a retention time of 3.66 minutes, and together with haemoglobin A₂ comprises 30.2% of total haemoglobin; peaks, from left to right, are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobins A₀ and haemoglobin E plus A₂ (shaded).

Fig. 5.21 Capillary electrophoresis (Sebia Capillarys 2) of a patient with haemoglobin E trait. The peaks from left to right are haemoglobins A, E and A₂. Note that haemoglobin A₂ separates from haemoglobin E.



gene and to a lesser extent to the XmnI polymorphism of the γ gene (*HBG2*).

Haemoglobin E is slightly unstable on heat and isopropanol stability tests.

Red cell protoporphyrin, sometimes used as a screening test for iron deficiency, can be elevated in haemoglobin E heterozygosity [52].

Osmotic fragility of red cells is reduced.

Pyrimidine 5' nucleotidase is reduced to levels comparable with those seen in heterozygotes for inherited deficiency [53].

Diagnosis

Diagnosis is dependent on demonstrating the presence of haemoglobin E and haemoglobin A by two independent techniques, with haemoglobin E being about a third of total haemoglobin. If the primary diagnostic method is cellulose acetate electrophoresis at alkaline pH, the differential diagnosis is mainly with haemoglobin C trait. If HPLC is the primary diagnostic method, the differential diagnosis is with heterozygosity for haemoglobin Lepore or other uncommon haemoglobins such as haemoglobin D-Iran (Fig. 5.22) and haemoglobin G-Coushatta (Fig. 5.23). The percentage of the variant and the precise retention time are useful. Although the

latter two variant haemoglobins elute in the E window, the shape of the chromatogram differs from that of haemoglobin E and the percentage is much higher.

DNA analysis can confirm the presence of the haemoglobin E allele, by the use of restriction enzymes, allele specific amplification or direct sequencing.

Screening tests for haemoglobin E are applicable in countries with a high prevalence of this variant haemoglobin. A one-tube osmotic fragility and a dichlorophenolindophenol (DCIP) test [54] have been used but the CMU-E test devised at the Chiang Mai University in Thailand has been found to have better sensitivity (100%) and specificity (99%) [55].

In the context of antenatal screening, it is important to exclude coexisting α^0 thalassaemia. It was found that a haemoglobin E of less than 22% by HPLC, an MCV of less than 72 fl and an MCH of less than 22.5 pg each had a sensitivity of 100% for identifying such cases, indicating the need for molecular studies [56]. Combining the haemoglobin E criterion with either of the other criteria improved specificity, in cases where iron deficiency was excluded, from 70.2–88.5% to 95.6%, reducing further the need for molecular analysis [56].

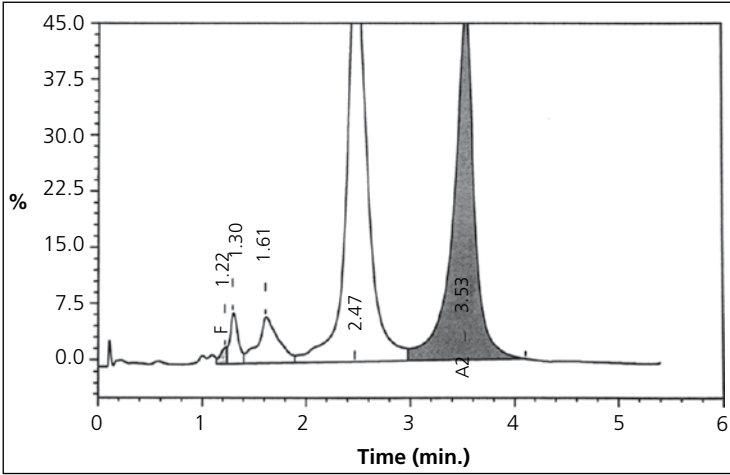


Fig. 5.22 HPLC chromatogram (Bio-Rad Variant II) from a patient with haemoglobin D-Iran heterozygosity; haemoglobin D-Iran appears in the A_2 window and, together with haemoglobin A_2 , comprised 46.6% with a retention time of 3.53 minutes; peaks, from left to right, are haemoglobin F, post-translationally modified haemoglobin A (two peaks), haemoglobin A_0 and haemoglobin D-Iran plus A_2 (shaded).

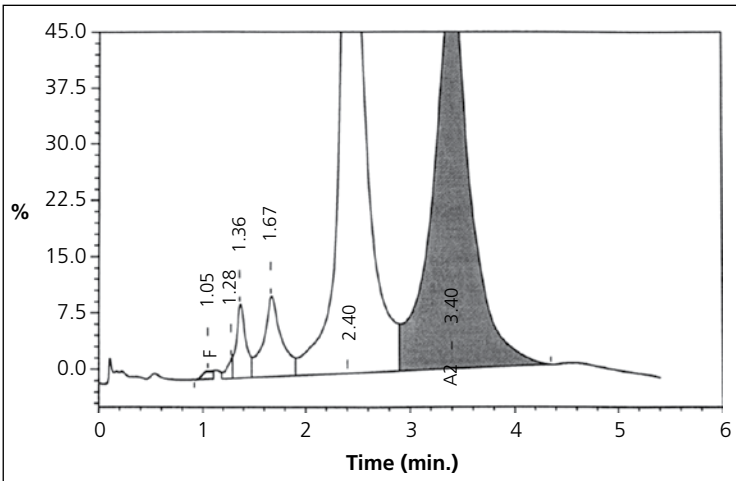


Fig. 5.23 HPLC chromatogram (Bio-Rad Variant II) from a patient with haemoglobin G-Coushatta heterozygosity; haemoglobin G-Coushatta appears in the A_2 window and, together with haemoglobin A_2 , comprised 51.5% with a retention time of 3.40 minutes; peaks, from left to right, are haemoglobin F, post-translationally modified haemoglobin A (two peaks), haemoglobins A_0 and haemoglobin G-Coushatta plus A_2 (shaded).

Haemoglobin E homozygosity (haemoglobin E 'disease')

Individuals with the genotype $\beta^E\beta^E$ are often completely asymptomatic. It has therefore been suggested that the term 'haemoglobin E homozygosity' is preferable to the term 'haemoglobin E disease'.

Clinical features

Reports suggest that usually, there is no anaemia or splenomegaly and rarely any evidence of haemolysis [30, 31, 57]. However, a study of 84 Indian patients found a mean Hb of 97 g/l, 31% splenomegaly, 12% hepatomegaly, 21%

cholelithiasis and jaundice as a frequent presenting feature [58].

Laboratory features

Blood count

The blood count often resembles that of β thalassaemia trait with a normal Hb or very mild anaemia, an increased RBC and reduced MCV and MCH. The MCHC is usually normal. The reticulocyte count is usually normal but may be slightly elevated. The MCV and MCH are significantly lower when there is coexisting α^0 thalassaemia heterozygosity but with overlapping values (one study showing MCV

mean values of 57.1 and 63.7 fl respectively and MCH mean values of 18 and 20.3 pg respectively) [59]. In the case of coinheritance of α^+ thalassaemia or haemoglobin Constant Spring, the MCV and MCH are reported to be higher [60].

Blood film

The blood film (Fig. 5.24) usually shows hypochromia and microcytosis with variable numbers of target cells and irregularly contracted cells.

Other investigations

Haemoglobin electrophoresis (Fig. 5.25), HPLC (Fig. 5.26) and capillary electrophoresis (Fig. 5.27) show the major haemoglobin to be haemoglobin E with haemoglobin E plus A_2 constituting 85–99% of total haemoglobin, the rest being haemoglobin F. Except when using capillary electrophoresis, haemoglobin A_2 is difficult to quantitate in the presence of haemoglobin E but with appropriate techniques can be shown to be somewhat increased; in one study, a mean value of 4.4% was observed in seven patients in comparison with a mean of 2.6% in control subjects [48]. Haemoglobin A_2 is significantly higher when there is coexisting α^0 thalassaemia heterozygosity, with mean values of 5.1 and 8.8% in one study; all subjects with coexisting α^0

thalassaemia had a haemoglobin A_2 of above 5.3%, whereas this was observed in 44% of subjects without coexisting α^0 thalassaemia [59]. Coexisting homozygosity for α^+ thalassaemia can also raise the haemoglobin A_2 [59]. Haemoglobin F after the age of five years may be normal or elevated, comprising up to 15% of total haemoglobin [41] but is usually less than

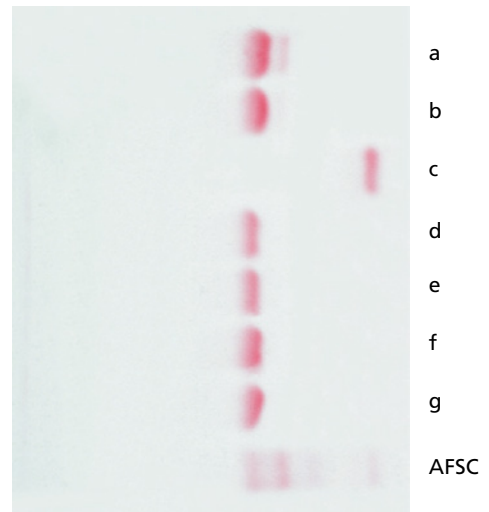


Fig. 5.25 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in a patient with haemoglobin E homozygosity (lane c); AFSC, control sample containing haemoglobins A, F, S and C (bands are faint).

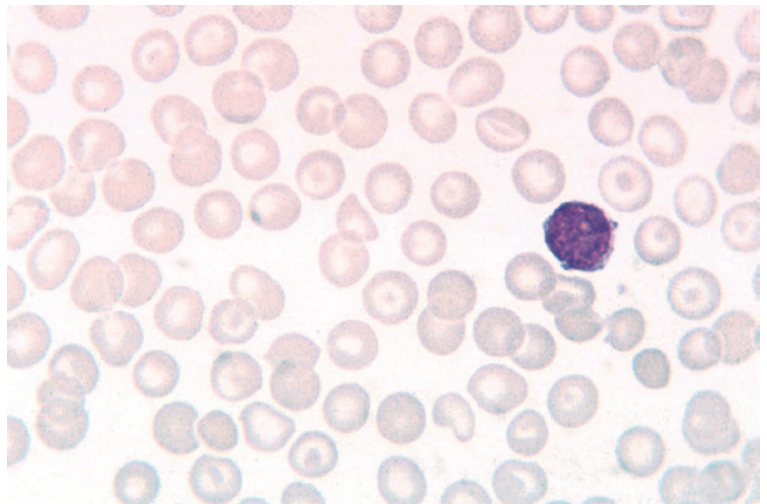


Fig. 5.24 Blood film in a patient with haemoglobin E homozygosity showing microcytosis, target cells and several irregularly contracted cells. MGG $\times 100$.

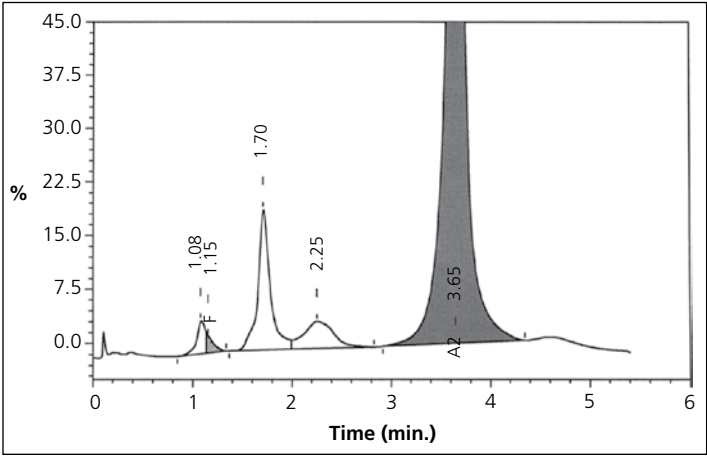


Fig. 5.26 HPLC chromatogram (Bio-Rad Variant II) from a patient with haemoglobin E homozygosity; haemoglobin E appears in the A_2 window with a retention time of 3.65 minutes, and together with haemoglobin A_2 comprised 94.1% of total haemoglobin. Note that haemoglobin E that has undergone post-translational modifications appears in the 'P3 window' and the A_0 windows; this should not be misinterpreted as indicating that haemoglobin A is present; peaks, from left to right, are haemoglobin F, post-translationally modified haemoglobin E (two peaks) and haemoglobin E plus A_2 (shaded).

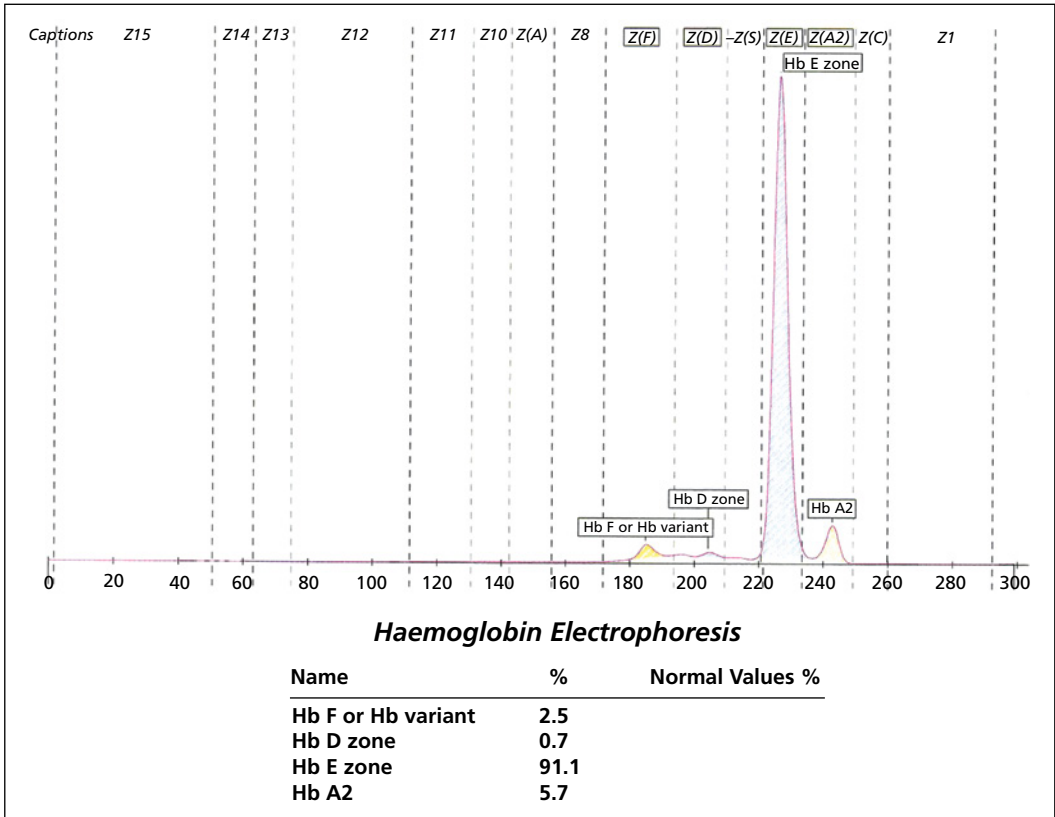


Fig. 5.27 Capillary electrophoresis (Sebia Capillarys 3) in haemoglobin E homozygosity showing haemoglobins F, E and A_2 . Note that haemoglobin A_2 is elevated and that, in contrast to compound heterozygosity for haemoglobin E and β^0 thalassaemia, there is only a trivial increase in haemoglobin F.

10%. However, a study of more than 200 Laotian children under the age of two years found that although on average haemoglobin F was 6%, up to 43% was observed [60]. Haemoglobin F levels greater than 10%, after five years of age, raise the possibility of haemoglobin E/ β thalassaemia being the underlying diagnosis. The percentage of haemoglobin F is decreased by coexisting α thalassaemia trait and if associated with XmnI polymorphism in the G γ gene, two polymorphisms in HBS1L-MYB and by a polymorphism of *BCL11A* [61]. Red cell life span is normal [30]. Osmotic fragility is reduced. The α :non- α chain synthesis ratio is increased, with reported ratios of 2.1 and 2.2 [30] and 1.59 and 1.61 [57]. The oxygen dissociation curve is slightly right-shifted [30], probably as a result of increased intracellular 2,3-diphosphoglycerate (2,3-DPG) since purified haemoglobin E has a normal oxygen affinity. Ultrastructural examination shows precipitated α chains in a proportion of late erythroblasts and bone marrow reticulocytes [62]. In one study, hyperbilirubinaemia was observed in 28% of patients and was found to correlate for homozygosity for a *UGT1A1* polymorphism [58]. Lactate dehydrogenase (LDH) was often increased, this together with the frequent observation of cholelithiasis suggesting haemolysis was at least a contributory factor to hyperbilirubinaemia in this population [58].

Diagnosis

The diagnosis requires identification of haemoglobin E as the sole variant haemoglobin, in the absence of haemoglobin A, by two independent techniques. The differential diagnosis is with haemoglobin E/ β^0 thalassaemia compound heterozygosity and requires assessment of clinical features and of the relative proportions of haemoglobins E, F and, if possible, A₂, family studies and DNA analysis.

Problems and pitfalls

Because of the genetic significance, it is essential to distinguish homozygosity for haemoglobin E from haemoglobin E/ β^0 thalassaemia, and to identify coexisting α^0 thalassaemia heterozygosity.

Haemoglobin E/ β thalassaemia

Haemoglobin E may be coinherited with either β^0 or β^+ thalassaemia [12, 63–66]. The compound heterozygous state is quite common in Thailand and surrounding countries but also occurs throughout a large part of South-East Asia, stretching from Indonesia to Sri Lanka, north-east India and Bangladesh, making this the most common cause of clinically significant thalassaemia in the world.

Haemoglobin E-Saskatoon should be distinguished from haemoglobin E; it does not interact with β thalassaemia [25].

Clinical features

The severity of compound heterozygosity for haemoglobin E and β thalassaemia is very variable, with the clinical picture ranging from that of mild thalassaemia intermedia to thalassaemia major. Most patients have a disease that is at least moderately severe. The variation in severity is not always readily explicable although it is in part related to the presence or absence of haemoglobin A and, if haemoglobin A is present, to its quantity. A high haemoglobin F or coinheritance of α thalassaemia trait or haemoglobin Constant Spring can ameliorate the condition [65, 67], whereas inheritance of triple α increases the severity [67]. For non-deletional hereditary persistence of fetal haemoglobin, homozygosity is required for the condition to be ameliorated [66]. The severity is also increased if a greater proportion of mRNA is alternatively spliced [41]. Methaemoglobin is increased, on average to 2.7%, the level correlating with disease severity and previous splenectomy and possibly being related to ascorbic acid deficiency [68].

The most severely affected individuals are transfusion dependent and have hepatosplenomegaly, intermittent jaundice, growth retardation, delayed sexual maturation and overexpansion of the bone marrow cavity, leading to facial deformity and malpositioned teeth; there is an increased incidence of gallstones, leg ulcers (seen in up to a fifth of patients [69]), osteoporosis and osteomalacia. Overexpansion of the

bone marrow cavity contributes to high output cardiac failure. There is a high rate of thromboembolism and hypoxaemia also occurs in splenectomised patients, possibly as the result of platelet aggregates reaching the lungs [41]. Non-splenectomised patients can develop pulmonary hypertension and right heart failure but splenectomy aggravates the prothrombotic state. Iron overload can cause diabetes mellitus and liver fibrosis. Less severely affected individuals may have splenomegaly and facial deformity but do not require regular transfusions to maintain life; however, these patients with the phenotype of non-transfusion-dependent thalassaemia can nevertheless develop extramedullary haemopoiesis, gallstones, osteoporosis, pulmonary hypertension and, less often, leg ulcers or hypogonadism [70]. Extramedullary haemopoiesis has sometimes led to compression of the spinal cord or brain by tumour-like masses of haemopoietic tissue, leading to paraplegia or convulsions. Hypersplenism can occur. Rarely *moya moya* has been reported [71]. Occasional patients are only mildly affected. During pregnancy or intercurrent infection, patients who are not usually transfusion dependent may become sufficiently anaemic to require transfusion.

In resource-poor settings where magnetic resonance imaging (MRI) is not readily available, serum ferritin can be useful for monitoring possible iron overload and reducing the need for MRI. In a Thai study of transfusion-dependent and transfusion-independent thalassaemia, predominantly E/ β thalassaemia, it was found possible to *diagnose* liver iron overload with cut-off points of >2500 and >1700 $\mu\text{g/l}$, respectively, in the two groups and to *exclude* cardiac iron overload with cut-off points of <2500 and <3000 $\mu\text{g/l}$ respectively [72].

An analysis of 50 British patients with haemoglobin E/ β thalassaemia (half of Bangladeshi origin, a quarter Indian/Pakistani and a quarter originating in South-East Asia) gives an idea of the usual degree of severity of this condition [73]. Half the patients were regularly transfused and nearly half had required splenectomy. A smaller number of US patients had disease of similar severity [73]

whereas of 22 Canadian patients, 30% were regularly transfused and 17% had required splenectomy [74]. In Thailand, patients are generally only transfused when this appears essential so that the natural history of the untreated disease is more readily apparent; about half of affected individuals have a thalassaemia major phenotype and half a thalassaemia intermedia phenotype. Of 76 Thai patients, 14% were considered to have a 'mild' phenotype, 55% a moderately severe phenotype and 30% a severe phenotype [41]. However, it should be noted that classification as 'mild' was possible with transfusion independence but with an Hb as low as 76 g/l; most of these patients would have to be regarded as having thalassaemia intermedia. Of 78 Indian patients, 37% had mild disease, 19% moderate (some becoming transfusion dependent later in life) and 44% severe (transfusion dependent) [75].

Hydroxycarbamide (hydroxyurea) therapy may ameliorate the disease by increasing haemoglobin F synthesis, and is used in combination with thalidomide in some countries. Patients who respond either show a reduction in transfusion requirement or become transfusion independent [76].

Laboratory features

Blood count

The Hb is lower than in haemoglobin E disease. It varies from 25 to 130 g/l, with the average being around 70–80 g/l in comparison with averages of around 95–115 g/l in patients with homozygosity for haemoglobin E. Because chain synthesis is more balanced, the Hb is higher in those who coinherit α thalassaemia; for example, in a study of non-transfusion-dependent Thai patients mainly with E/ β^0 thalassaemia, the mean Hb was 76 g/l in 42 patients with four α genes in comparison with 119 g/l in a patient with homozygous α^+ thalassaemia and a mean of 105 g/l in three patients with heterozygous α^0 thalassaemia [77]. The MCV and MCH are, on average, more reduced than in haemoglobin E trait. The MCHC is reduced and

the RDW is increased. The reticulocyte count is slightly increased (e.g. 4–6%).

Hydroxycarbamide therapy is associated with a slight rise in Hb (usually less than 10 g/l rise), the expected rise in the MCV and MCH and an appreciable fall in the reticulocyte count [78].

Blood film

The blood film (Figs 5.28 and 5.29) shows anisocytosis, poikilocytosis, hypochromia, microcytosis, target cells, irregularly contracted cells and NRBC. There may be basophilic

stippling. If splenectomy has been undertaken there will be Heinz body-like α chain inclusions [79], in addition to the usual post-splenectomy changes.

Other investigations

Haemoglobin electrophoresis and HPLC show haemoglobins E/A₂ and F in the case of haemoglobin E/ β^0 thalassaemia and A, E/A₂ and F in the case of haemoglobin E/ β^+ thalassaemia. When haemoglobin A is present, it usually constitutes around 10% of total

Fig. 5.28 Blood film of a six-year-old boy with haemoglobin E/ β^+ thalassaemia showing anisocytosis, target cells and irregularly contracted cells. There is one cell with the haemoglobin condensed into one third of the cell. The red cells indices were RBC $3.97 \times 10^{12}/l$, Hb 60 g/l, MCV 54.7 fl and MCH 15.1 pg. Haemoglobin A was 10%. MGG $\times 100$.

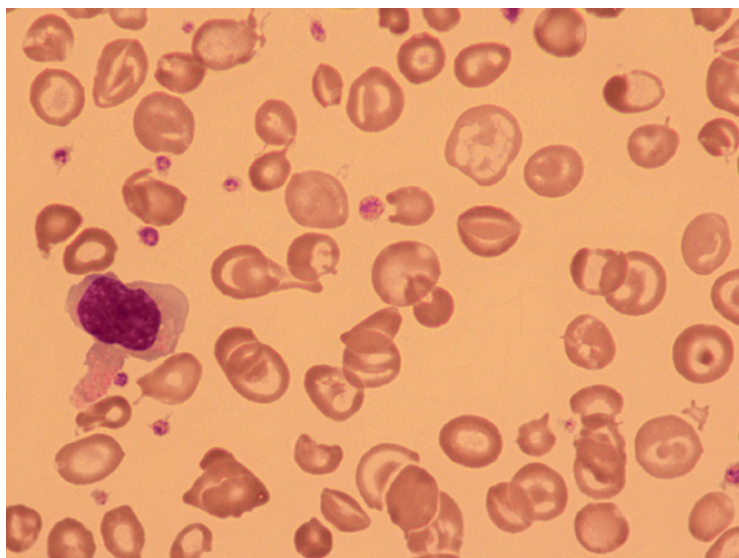
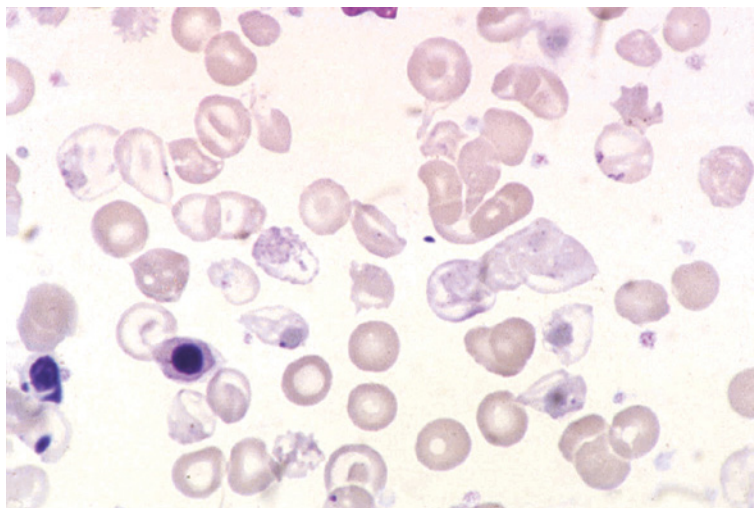


Fig. 5.29 Blood film of a patient with haemoglobin E/ β thalassaemia compound heterozygosity severe enough to have required splenectomy showing poikilocytosis, some hypochromic cells, Pappenheimer bodies, a nucleated red blood cell and erythrocytes containing precipitates that are likely to represent precipitated α chain. MGG $\times 100$.



haemoglobin. Studies in Thailand have shown that compound heterozygotes for E and β^0 thalassaemia usually have haemoglobin E/ A_2 representing 30–60% of total haemoglobin; there is little overlap with homozygous E where the percentage is usually $\geq 65\%$ [80]. Conversely, haemoglobin F is usually $\geq 15\%$ in E/ β^0 thalassaemia whereas in EE it is less than 15% and usually less than 5% [80]. However, some patients with E/ β^0 thalassaemia have a higher haemoglobin E (e.g. $>75\%$ and haemoglobin F $<15\%$) [80]. In 56 non-transfusion-dependent Sri Lankan patients, the mean level of haemoglobin F was 28% with a range of 6–51% [81]. The percentage of haemoglobin F is influenced by coinheritance of α thalassaemia; in a study of Thai patients, there was a mean haemoglobin F of 33% in patients with four α genes in comparison with mean levels of 25.7% in 20 patients with $-\alpha^{3.7}/\alpha\alpha$ [77]. Haemoglobin F percentage is also influenced by polymorphisms affecting haemoglobin F synthesis [77]. The increase in haemoglobin F is mainly attributable to erythropoietin-driven erythroid hyperplasia and possibly increased F-cell production combined with preferential survival of F cells [82]. It is not surprising that, overall, haemoglobin F is very variable, from 5% to 87% [64].

When haemoglobins are quantified by capillary electrophoresis, permitting haemoglobin A_2 to be distinguished from E, the findings are somewhat different. Haemoglobin E is approximately 82–95% in E homozygotes and approximately 78–80% in E/ β^0 thalassaemia [83]. Haemoglobin A_2 was found to be above 6% in E/ β^0 thalassaemia and was above this level in only one of 19 E homozygotes [83]. Surprisingly, in this study haemoglobin F was higher in EE (around 2–12%) than in compound heterozygotes (around 2–8%), but the difference was not significant [83]. The MCV was above 55 fl in 18 of 19 EE and in only one of nine E/ β^0 thalassaemias while the Hb was higher in EE (around 85–150 g/l) than in compound heterozygotes (around 62–98 g/l).

The formula $(7.3 \times \text{Hb}A_2) + \text{HbF}$ has been found useful to screen for E/ β^0 thalassaemia with a score above 60 having 100% sensitivity

and 96.5% specificity in adults [84]. Sensitivity appears to be equally good in children but with specificity being much lower in children under the age of two years than in adults, since haemoglobin F is higher in young children with E homozygosity than it is in adults [85]. Nevertheless, application of this formula considerably reduces the number of cases needing molecular testing. E/ $\delta\beta^0$ thalassaemia is characterised by a higher haemoglobin F (mean 55%) and a low haemoglobin A_2 (mean 2.4%) [84].

Because of the variability in haemoglobin F, it is prudent to perform DNA analysis when haemoglobin E is more than 75% and haemoglobin F is between 5% and 15%; in high prevalence areas, if capillary electrophoresis is used and a haemoglobin A_2 percentage is therefore available, the need for molecular analysis can be reduced by use of the above formula.

Tests for haemoglobin instability are weakly positive. Osmotic fragility and red cell life span are reduced. There is a marked increase in 2,3-DPG with a resultant marked increase in P_{50} (despite the increased percentage of haemoglobin F), which means that quite severe anaemia is well tolerated [81]. Erythropoietin concentration is markedly increased [81]. The bone marrow shows erythroid hyperplasia, dyserythropoiesis, increased macrophage activity and increased storage iron. Serum transferrin receptor concentration is increased, reflecting expanded erythropoiesis.

Hydroxycarbamide therapy is associated with a significant rise in haemoglobin F percentage and a reciprocal fall in haemoglobin E percentage [78]. There is an increase in Hb, MCV and MCH and a fall in serum ferritin and plasma bilirubin [76]. Regular blood transfusion is associated with a greater decrease in haemoglobin F synthesis than in haemoglobin E synthesis [82].

Diagnosis

The differential diagnosis is as for haemoglobin E homozygosity (see earlier).

Coinheritance of haemoglobin E with other variant haemoglobins, thalassaemias and haematological disorders

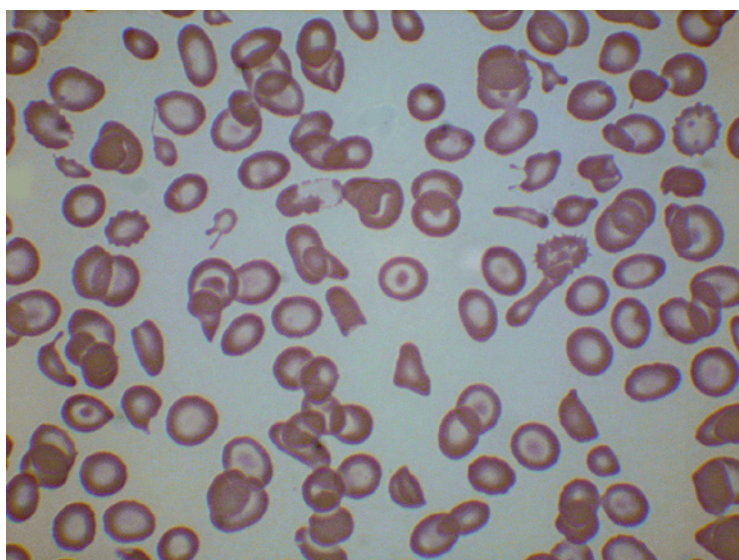
Coinheritance of haemoglobins S and E has been described on page 273 and the interaction with β thalassaemia, α thalassaemia trait and haemoglobin C in this chapter.

Because both are relatively common in South-East Asia, haemoglobin E heterozygosity or homozygosity may coexist with the genotype of haemoglobin H disease. In one series of patients, individuals with heterozygosity for haemoglobin E and the genotype of haemoglobin H disease had a condition that was clinically similar to uncomplicated haemoglobin H disease, with the average Hb being around 70–80 g/l and the MCV averaging 67 fl [86]. In another series, the Hb was around 70–90 g/l (similar to uncomplicated haemoglobin H disease) but the MCV was lower at 52–58 fl [87]. In addition to the blood film features of haemoglobin H disease (microcytosis and marked poikilocytosis), there can also be irregularly contracted cells and erythrocytes with the haemoglobin retracted to one or both ends of the cell (Fig. 5.30) [88]. The haemoglobins present are A, E and Bart's, with there being more A than E and more E than Bart's [89]. Only a small percentage of cells

(1–5%) [86], if any [87], contain haemoglobin H inclusions and haemoglobin H is usually not detected on electrophoresis [87]. Haemoglobin F is increased to 3–13% whereas in uncomplicated haemoglobin H disease it averages 1–2% [87]. It appears that the reduced quantity of α chain combines preferentially with the normal β chain rather than with β^E [86] with the percentage of haemoglobin E plus A_2 being as low as 8–16% [87]. The designation EABart's disease has been used. Coinheritance of haemoglobin E heterozygosity and $--/\alpha^{CS}\alpha$ (Fig. 5.31) [90] or $--/\alpha^{PS}\alpha$ has a more severe clinical phenotype than coinheritance of haemoglobin E heterozygosity and $--/-\alpha$ [41] with the Hb being on average about 20 g/l lower [91] and with haemoglobin H inclusions being found in around 5% of erythrocytes.

Homozygosity for haemoglobin E coinherited with the genotype of haemoglobin H disease gives a severe thalassaemia intermedia phenotype with an Hb similar to that in AEBart's disease but on average a lower MCV (mean 61 fl) (Fig. 5.32). Haemoglobins present are usually E, Bart's and F, comprising about 80%, 1–25% (average 10%) and 1–7% respectively of total haemoglobin [86, 92]. No inclusions are detected in haemoglobin H preparations, suggesting that the β^E chains do not form tetramers. The designation EFBart's disease has

Fig. 5.30 Blood film of a Thai patient with haemoglobin E heterozygosity and the genotype of haemoglobin H disease showing microcytosis, marked poikilocytosis (including target cells and schistocytes) and an erythrocyte with haemoglobin retracted to one end of the cell. The full blood count showed RBC $5.34 \times 10^{12}/l$, Hb 88 g/l, MCV 52 fl, MCH 16.5 pg, MCHC 314 g/l. MGG $\times 100$.



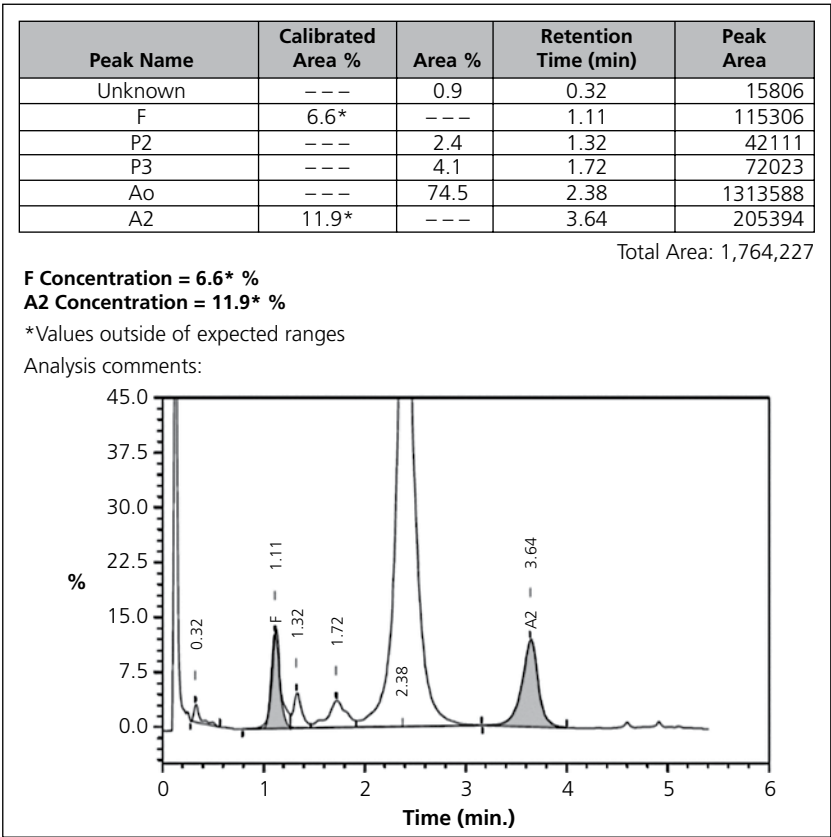


Fig. 5.31 HPLC (Bio-Rad Variant II) of a Thai man with haemoglobin E heterozygosity and the genotype of non-deletional haemoglobin H disease showing, from left to right, haemoglobin Bart’s, very small peaks of altered haemoglobin F, haemoglobin F, two peaks of post-translationally modified A, haemoglobin A₀, haemoglobin E plus A₂, and two small peaks representing haemoglobin Constant Spring. (With thanks to Dr Sunisa Kongkiatkamon, Bangkok.)

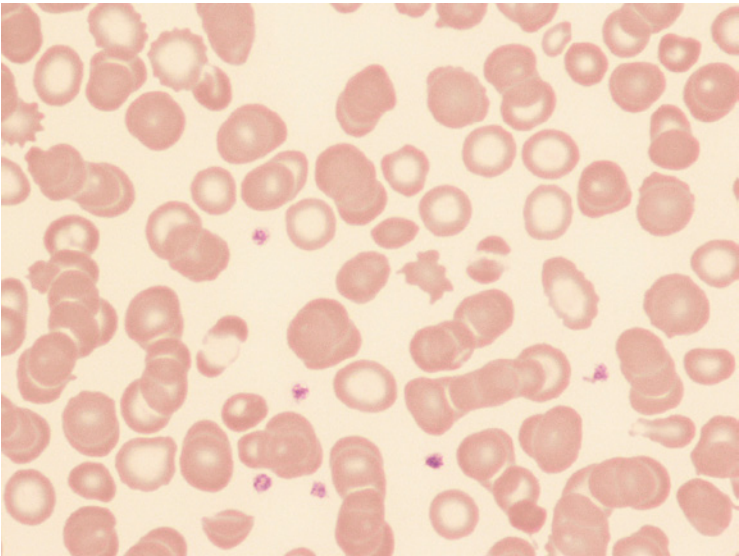


Fig. 5.32 Blood film of a patient with haemoglobin E homozygosity and the genotype of haemoglobin H disease ($\alpha^{-3.7} / -_{SEA}$) showing hypochromia, microcytosis and poikilocytosis with some erythrocytes showing retraction of haemoglobin to the two ends of the cells. Red cell indices were RBC $6.29 \times 10^{12}/l$, Hb 97 g/l, MCV 55.3 fl, MCH 15.4 pg and MCHC 279 g/l.

been used but it should be noted that some patients have been described with no increase of haemoglobin F and with haemoglobin Bart's not being detected [92]. Closely related clinical phenotypes result from the coinheritance of either $\beta^E\beta^E$ or $\beta^E\beta^0$ and either $--/-\alpha$ or $--/\alpha^{CS}\alpha$. Although the α^{CS} gene is present in patients with coinheritance of $--/\alpha^{CS}\alpha$ and haemoglobin E homozygosity, no haemoglobin Constant Spring is detected [91].

Coinheritance of haemoglobin E heterozygosity and $--^{SEA}/\alpha\alpha$ is of genetic significance. It leads to a lower haemoglobin E plus A_2 percentage (mean 22.8% cf. 27.6%), lower MCV (68.6 cf. 75.7fl) and lower MCH (22.5 cf. 24.9pg) [93]. Algorithms have been devised to indicate which

patients require testing for α^0 thalassaemia in antenatal screening programmes; they differ according to the Hb [93].

Interaction of haemoglobin E and haemoglobin Lepore [25] can cause significant anaemia with the phenotype of thalassaemia intermedia.

Coinheritance of haemoglobin E and haemoglobin D-Punjab (Fig. 5.33) may be associated with mild anaemia and microcytosis; haemoglobin D is around 60–65% [94]. The condition is asymptomatic.

Coinheritance of haemoglobin E and deletional hereditary persistence of fetal haemoglobin leads to red cell indices similar to those of thalassaemia trait but no clinical abnormality [23].

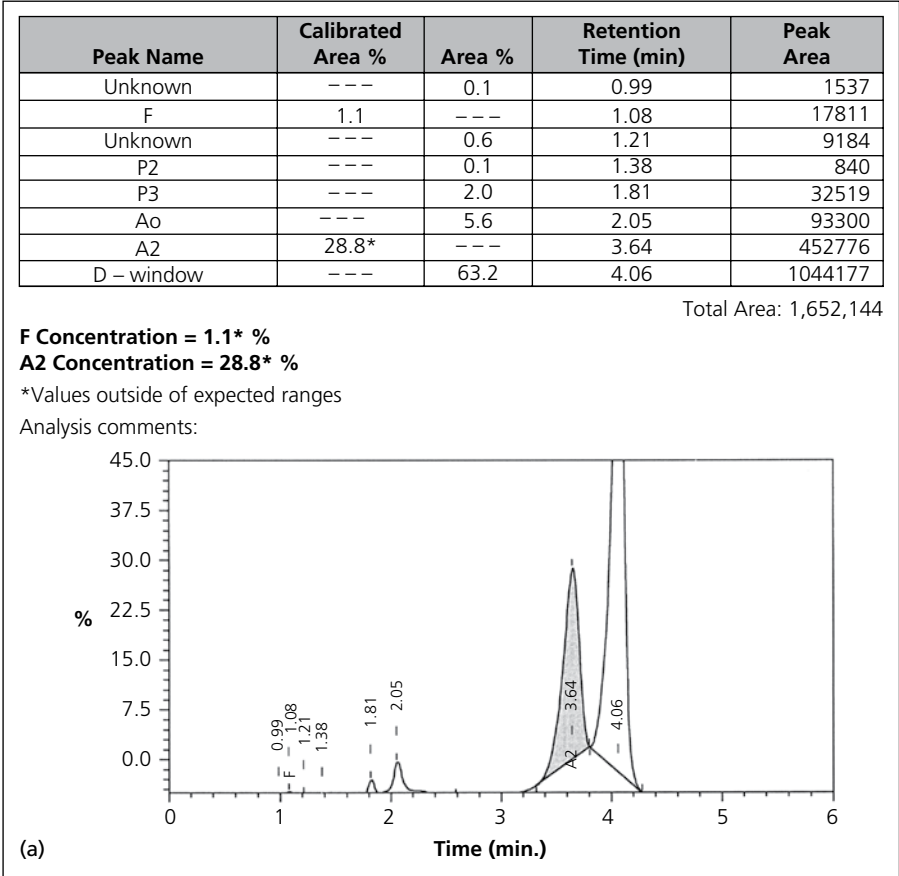


Fig. 5.33 Investigations of a patient with compound heterozygosity for haemoglobin E and haemoglobin D-Punjab: (a) HPLC (Bio-Rad Variant II) showing two major overlapping peaks representing haemoglobin E plus A_2 and haemoglobin D-Punjab; (Continued on p. 330.)

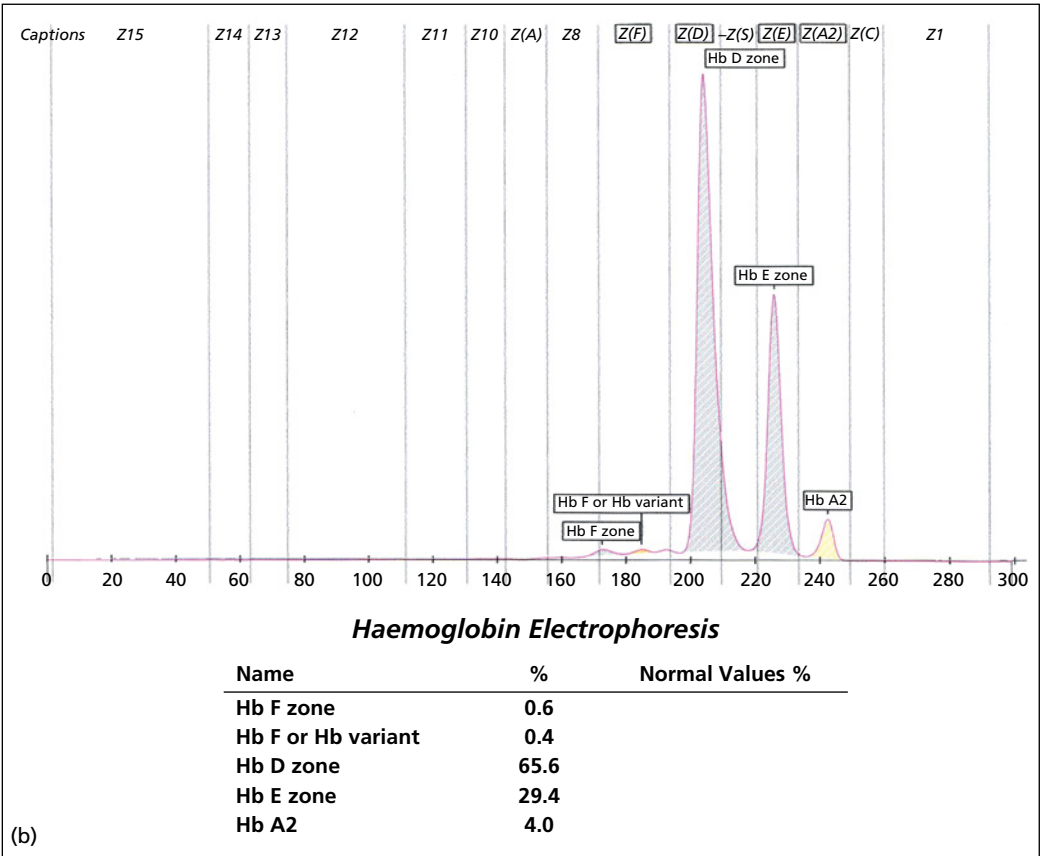


Fig. 5.33 *Continued.* (b) capillary electrophoresis (Sebia Capillaries) showing two major peaks representing haemoglobins D-Punjab and E and a minor peak of haemoglobin A₂ (4%).

Coinheritance of haemoglobin E and the elongated β chain variant, haemoglobin Tak, has been reported. A mild polycythaemia was observed in one patient [89].

A single individual has been reported in whom coinheritance of haemoglobin E disease and pyrimidine 5' nucleotidase deficiency led to a clinically severe phenotype with reticulocytosis and a haemoglobin concentration of less than 30 g/l, which increased significantly following splenectomy [73]. The mechanism is a marked increase in haemoglobin E instability.

Haemoglobin D-Punjab/D-Los Angeles

Haemoglobin D-Punjab is a β chain variant, $\alpha_2\beta_2^{121 \text{ Glu} \rightarrow \text{Gln}}$, initially described under the

name of haemoglobin D-Los Angeles. The latter could be regarded as the correct designation and is used in North America whereas in the UK the designation D-Punjab tends to be used. This variant haemoglobin has also been designated haemoglobin D-Cyprus, D-Conley, D-Chicago, D-North Carolina, D-Portugal and haemoglobin Oak Ridge [95]. Its only major importance is because of its interaction with haemoglobin S (see page 266). Its highest incidence is among Sikhs in the Punjab who show a prevalence of 2–3%. Gujaratis have a prevalence of about 1% and a similar prevalence is found in British Pakistanis. There is a low but significant prevalence among African Americans (0.4%) [96] and African Caribbeans (0.01) [33]. It has also been reported in individuals with

American Indian ancestry. There is a low prevalence among white populations in England, France and Portugal who have had a close relationship with India. In England the highest prevalence is in Norfolk where it has been attributed to the sojourn of the IXth foot regiment in India. Haemoglobin D-Punjab is also found in Pakistan, Afghanistan, Iran, China, Turkey, the Balkans, Holland, Australia and North Africa. The prevalence in Sicily is 0.5% [97] and in Sri Lanka is 0–1.3% in different districts [38]. There is also a variant haemoglobin, haemoglobin Cleveland, in which the haemoglobin D mutation is one of two mutations.

It is important to distinguish haemoglobin D-Punjab from other α and β chain variants with similar electrophoretic properties but with less or no clinical significance. Other β chain variants with similar electrophoretic mobility to haemoglobin D-Punjab on cellulose acetate at alkaline pH include haemoglobin D-Ibadan, $\alpha_2\beta_2^{87 \text{ Thr} \rightarrow \text{Lys}}$, haemoglobin D-Iran (see Fig. 5.15), $\alpha_2\beta_2^{22 \text{ Glu} \rightarrow \text{Gln}}$, haemoglobin G-Coushatta (see Fig. 5.16), $\alpha_2\beta_2^{22 \text{ Glu} \rightarrow \text{Ala}}$ and haemoglobin Korle-Bu, $\alpha_2\beta_2^{73 \text{ Asp} \rightarrow \text{Asn}}$, none of which appears to be of clinical significance. Haemoglobin Korle-Bu, initially described as haemoglobin G and then as haemoglobin G-Accra, is most common in West Africa (Ghana and the Ivory Coast) [98]. It moves slightly on the A side of S on electrophoresis at acid pH and has the same retention time as haemoglobin A₂ on HPLC. The most common α chain variant with the potential to be confused with haemoglobin D-Punjab is haemoglobin G-Philadelphia (see later).

Haemoglobin D has a normal stability and a normal or slightly increased oxygen affinity.

Haemoglobin D-Punjab trait

Heterozygosity for haemoglobin D-Punjab is of genetic but no other clinical significance.

Clinical features

Haemoglobin D heterozygotes are asymptomatic.

Laboratory features

Blood count

The blood count is normal [99], unless there is coexisting α thalassaemia trait. The reticulocyte count is normal.

Blood film

The blood film may be normal or show some target cells.

Other investigations

On cellulose acetate at alkaline pH, haemoglobin D-Punjab has the same electrophoretic mobility as haemoglobin S and a variety of other α and β chain variants randomly designated haemoglobin D or G. On citrate agar or agarose gel at acid pH, haemoglobin variants designated D or G separate from S and move with or near to haemoglobin A; D/G variants cannot be separated from each other by either of these electrophoretic techniques. On isoelectric focusing D-Punjab is easily separated from haemoglobins A and S and from the other relatively common D/G variants, including G-Philadelphia. It can be separated from S, A, G-Ibadan, G-Coushatta, D-Iran and haemoglobin Korle-Bu by HPLC (Fig. 5.34). On capillary electrophoresis, haemoglobin D-Punjab separates from haemoglobins A and S and appears in the 'D zone' as do haemoglobins G-Philadelphia and Lepore (Fig. 5.35). Although G-Philadelphia elutes in the D-Punjab window, the two can be distinguished on HPLC by the presence of the minor G₂ fraction in association with haemoglobin G-Philadelphia. A distinction can similarly be made on capillary electrophoresis.

In heterozygotes, haemoglobin D is somewhat less than 50% of total haemoglobin. Coexisting α thalassaemia reduces the percentage of the variant [100].

Osmotic fragility and red cell survival are normal.

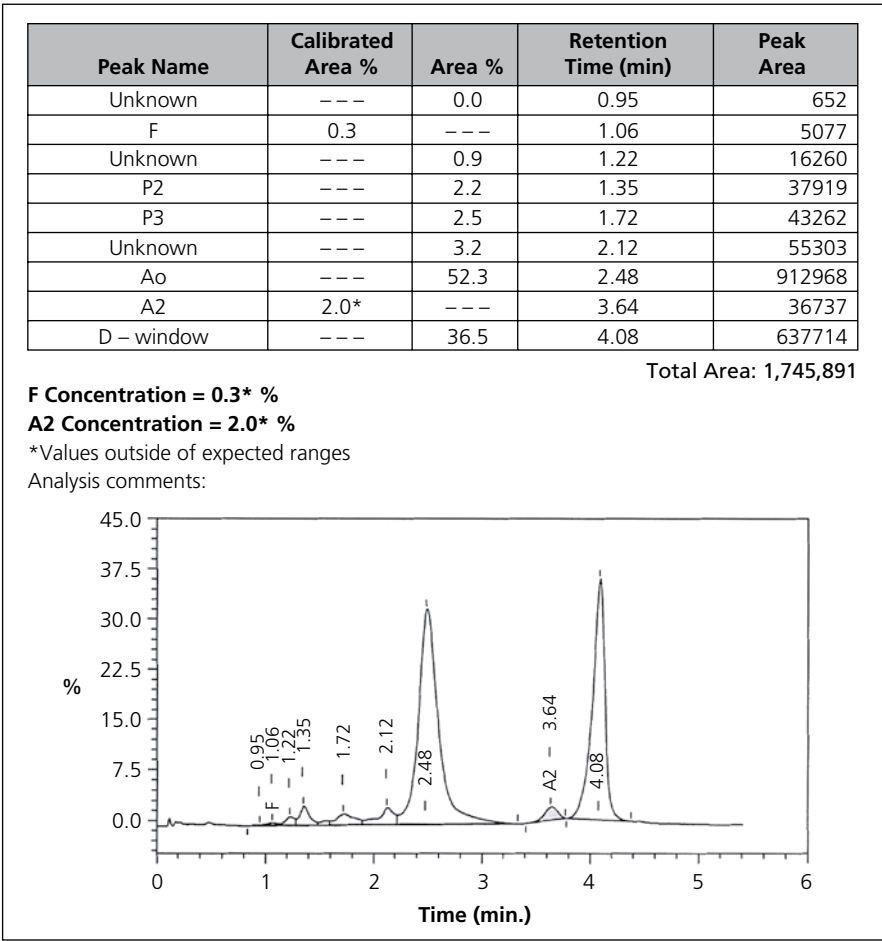


Fig. 5.34 HPLC (Bio-Rad Variant II) in haemoglobin D-Punjab heterozygosity. The peaks from left to right are: injection artefact, haemoglobin F, four low peaks representing post-translationally modified haemoglobins A and D, haemoglobin A_v, haemoglobin A₂ (shaded) and haemoglobin D-Punjab.

Diagnosis

Diagnosis requires the identification of haemoglobins A and D-Punjab by two independent techniques. It should be noted that a reliable identification of haemoglobin D-Punjab by a combination of cellulose acetate electrophoresis at alkaline pH and acid agarose electrophoresis is not possible. Isoelectric focusing and HPLC are more useful. Confirmation by DNA analysis is possible.

Haemoglobin A₂ is underestimated by HPLC in the presence of haemoglobin D whereas it is

appears to be accurate by capillary zone electrophoresis [47, 49].

Haemoglobin D-Punjab disease

Haemoglobin D-Punjab homozygosity (haemoglobin D disease) is associated with a clinically mild phenotype.

Clinical features

There may be mild haemolysis and sometimes a mild haemolytic anaemia [99, 101, 102]. Some homozygotes have splenomegaly.

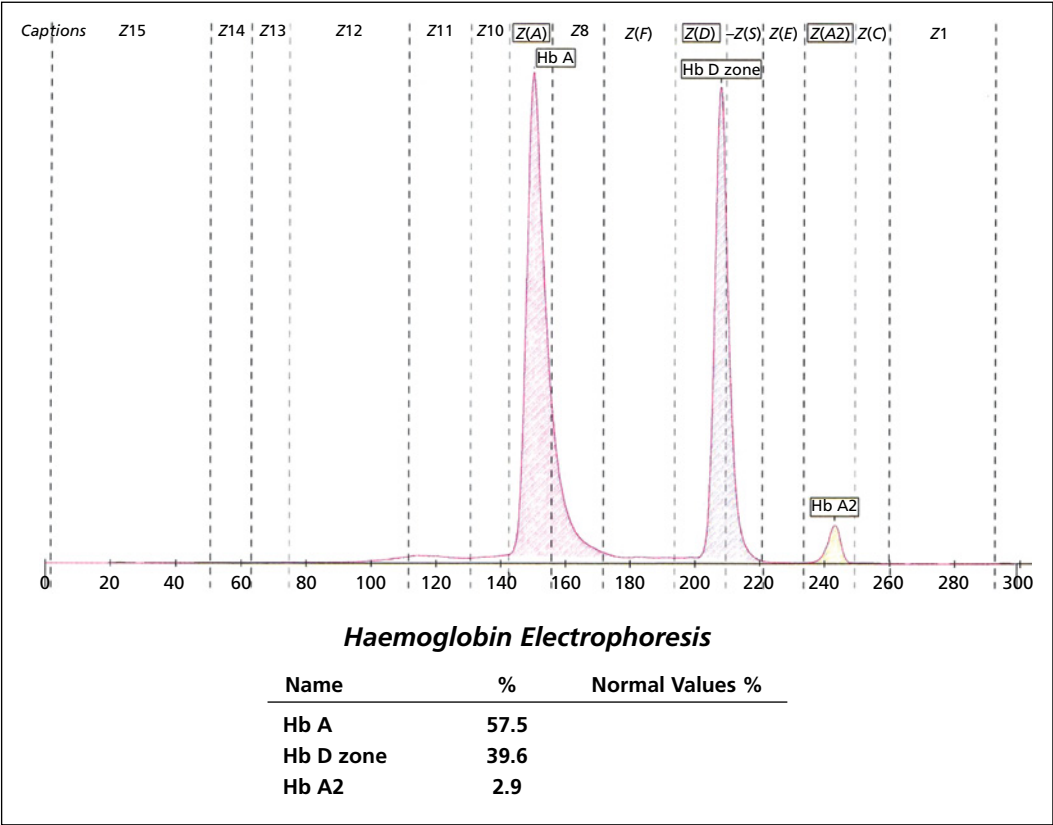


Fig. 5.35 Capillary electrophoresis (Sebia Capillarys 3) in haemoglobin D-Punjab heterozygosity. The peaks from left to right are: haemoglobin D, haemoglobin A and haemoglobin A₂.

Laboratory features

Blood count

Reported haemoglobin concentration has ranged from 90 to 100 g/l up to normal levels. The red cell indices may be suggestive of thalassaemia with an elevated RBC and reduced MCV and MCH. The reticulocyte count may be normal or elevated to 2–4%. The blood film shows infrequent to numerous target cells and may show irregularly contracted cells. Osmotic fragility is reduced. Red cell survival is slightly reduced. Haemoglobin D constitutes the major part of haemoglobin; haemoglobin A₂ and F are not increased.

Diagnosis

The diagnosis is dependent on identification of haemoglobin D Punjab as the sole variant

haemoglobin in the absence of haemoglobin A, by two reliable independent techniques (Figs 5.36 and 5.37). If there is microcytosis, the differential diagnosis is with compound heterozygosity for haemoglobin D-Punjab and β^0 thalassaemia and DNA analysis is required.

Haemoglobin D-Punjab/ β thalassaemia

Compound heterozygosity for haemoglobin D-Punjab and β^0 thalassaemia produces a mild thalassaemic condition [23, 103]. Haemoglobin D-Punjab/ β^+ thalassaemia [95, 104] is less common than haemoglobin D-Punjab/ β^0 thalassaemia. Curiously, one of the reported cases had a more severe thalassaemic phenotype than some of the cases of

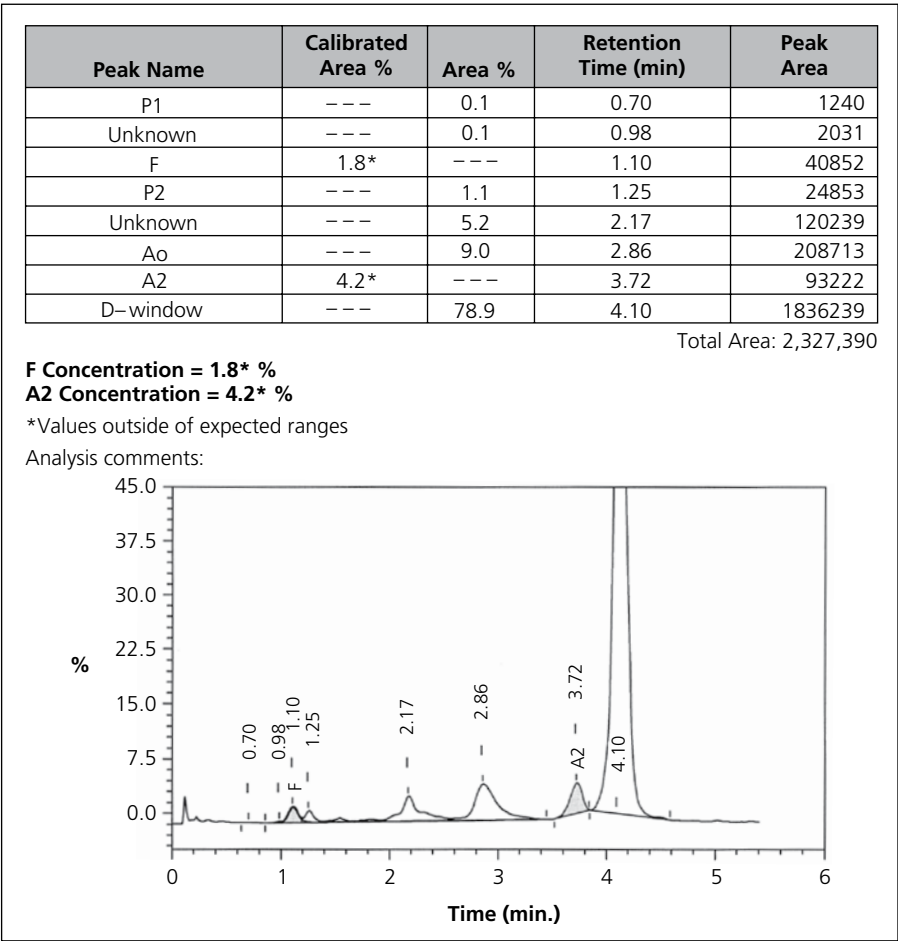


Fig. 5.36 HPLC (Bio-Rad Variant II) in haemoglobin D-Punjab homozygosity. The peaks from left to right are: injection artefact, haemoglobin F, four low peaks representing post-translationally modified haemoglobin D, haemoglobin A₂ (shaded) and haemoglobin D-Punjab.

haemoglobin D-Punjab/ β^0 thalassaemia [104]. In antenatal screening it is important to distinguish this compound heterozygous state from haemoglobin D-Punjab homozygosity; this is made more difficult by the apparently normal haemoglobin A₂ in some individuals [105].

Clinical features

Compound heterozygotes more often resemble β thalassaemia trait than β thalassaemia intermedia. There is mild anaemia and sometimes splenomegaly.

Laboratory features

Blood count

Reported Hb has usually ranged from 105 g/l up to normal levels but occasional patients have been more severely anaemic. The RBC may be elevated and there is marked reduction of the MCV and MCH. The reticulocyte count is slightly elevated.

Blood film

The blood film (Fig. 5.38) shows anisocytosis, poikilocytosis, hypochromia, numerous target cells and some irregularly contracted cells.

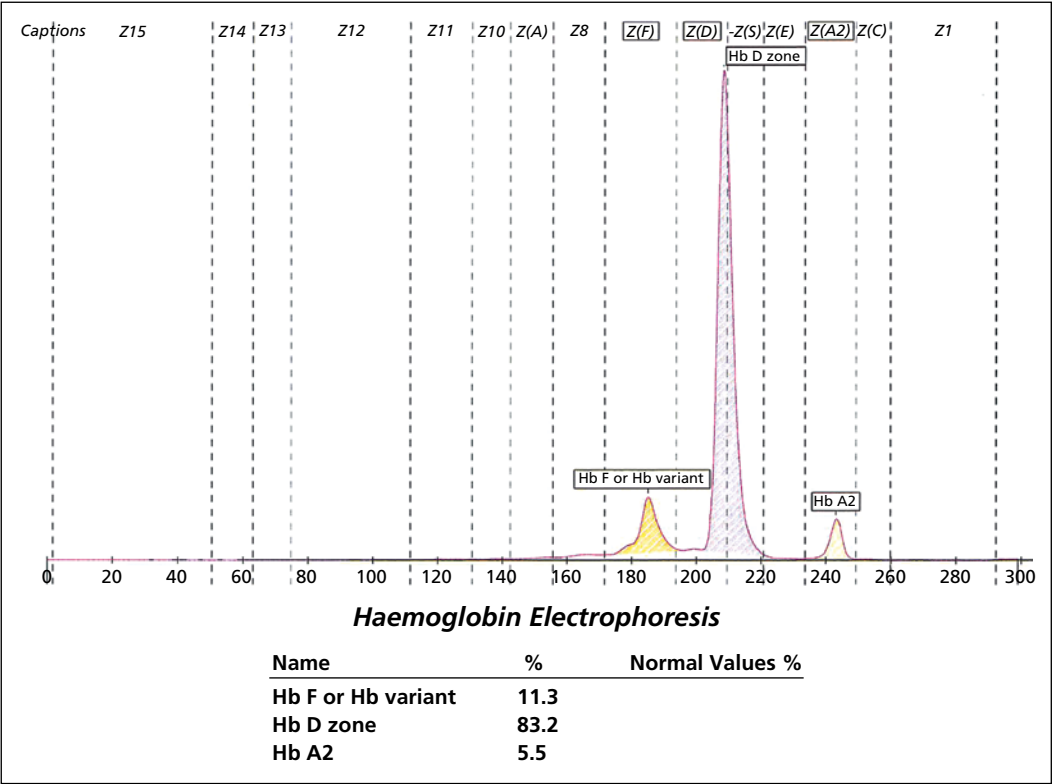


Fig. 5.37 Capillary electrophoresis (Sebia Capillars 3) in haemoglobin D-Punjab homozygosity. The peaks from left to right are: haemoglobin F, haemoglobin D and haemoglobin A₂.

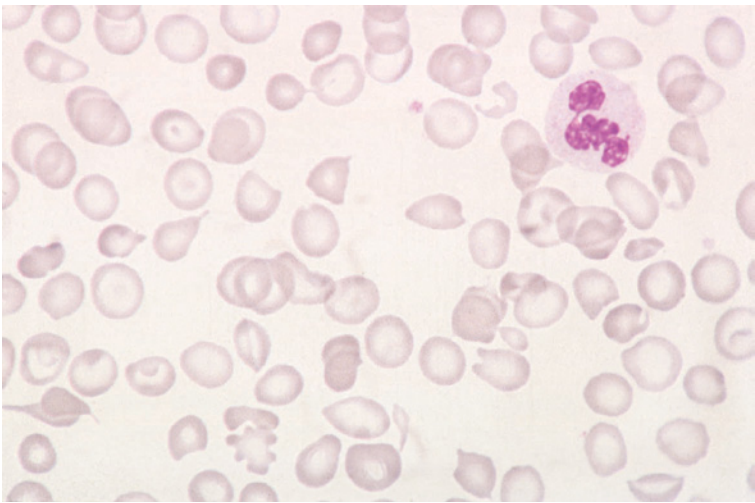


Fig. 5.38 Blood film in a patient with haemoglobin D-Punjab/ β^0 thalassaemia compound heterozygosity showing hypochromia, microcytosis and poikilocytosis, including target cells. MGG $\times 100$.

Other investigations

Haemoglobin electrophoresis and HPLC show almost all the haemoglobin to be haemoglobin D. Haemoglobin F is elevated in some patients. The haemoglobin A₂ percentage has been reported to range from high normal levels (3%) to levels similar to those seen in β thalassaemia trait (4.6–8%) [23, 25]. Reported normal levels probably result from underestimation of haemoglobin A₂ by HPLC in the presence of haemoglobin D-Punjab. This appears not to be a problem when microcolumn chromatography or capillary electrophoresis is used [47, 49, 105].

Coinheritance of haemoglobin D-Punjab and other variant haemoglobins and thalassaemias

Coinheritance of haemoglobin D-Punjab and haemoglobin S has been described on page 266. Coinheritance with haemoglobin G-Philadelphia is discussed below. A rare coinheritance of haemoglobin D-Punjab and haemoglobin O-Arab was associated with marked microcytosis and mild anaemia that was likely to be clinically silent [106]. Coinheritance with D-Iran (Fig. 5.39) and Q-India (Fig. 5.40) is observed.

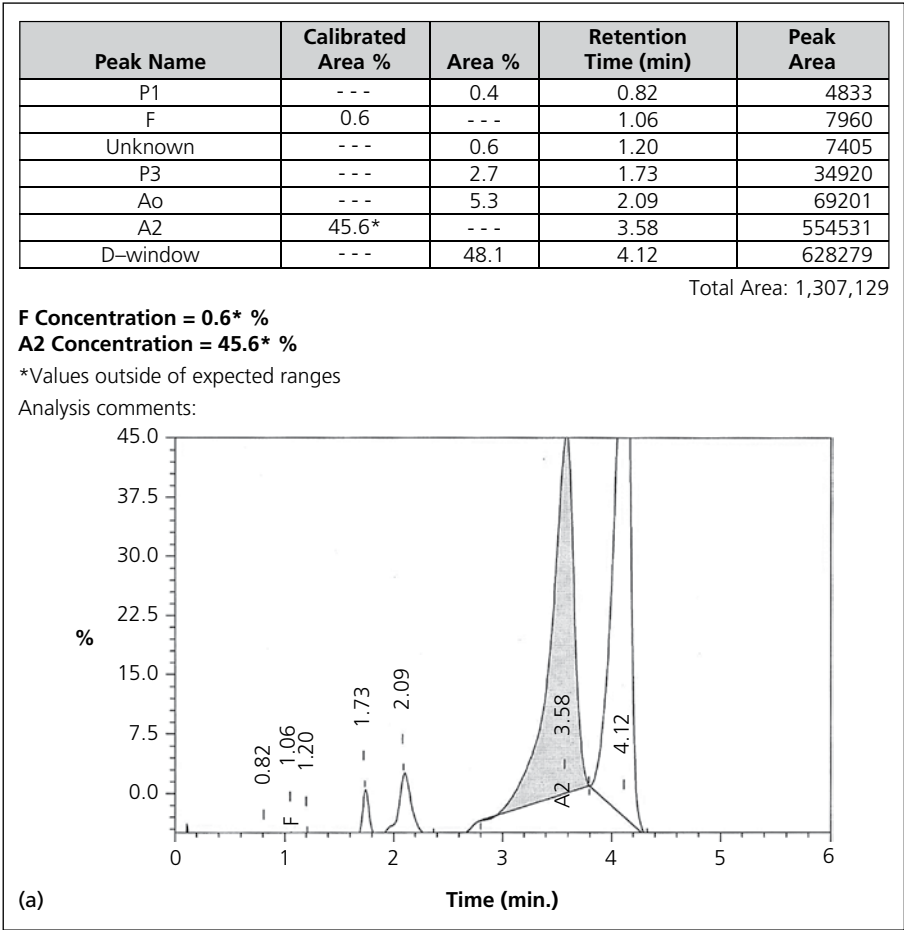


Fig. 5.39 Investigations of a patient who is a compound heterozygote for haemoglobin D-Punjab and haemoglobin D-Iran. (a) HPLC (Bio-Rad Variant II) showing two major overlapping peaks, haemoglobin D-Iran (in the haemoglobin A₂ window) and haemoglobin D-Punjab (in the D window);

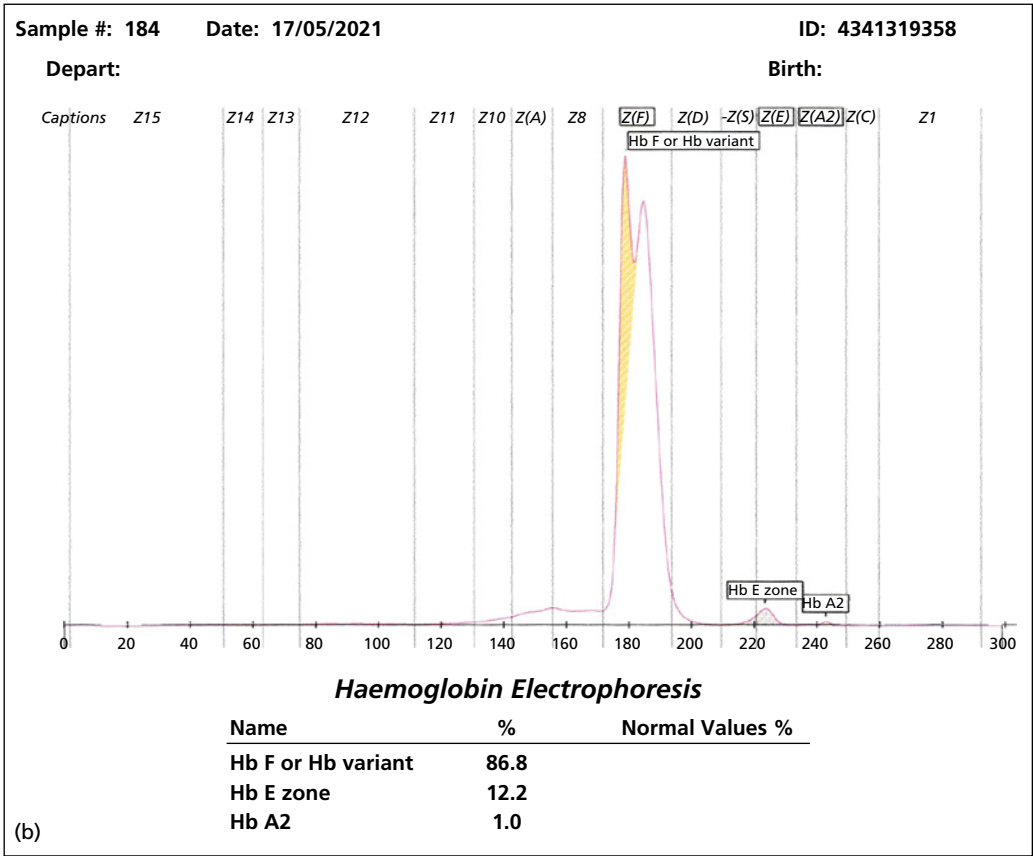


Fig. 5.39 Continued. (b) capillary electrophoresis (Sebia Capillarys) showing overlapping peaks in the F zone.

Diagnosis

The diagnosis and differential diagnosis are as for haemoglobin D-Punjab homozygosity.

Haemoglobin G-Philadelphia

Haemoglobin G-Philadelphia, $\alpha_2^{69 \text{ Asp} \rightarrow \text{Lys}}\beta_2$, is of no clinical significance but has the potential to cause diagnostic confusion, either when it occurs alone or when it is coinherited with other variant haemoglobins. This haemoglobin has also been designated haemoglobin Stanleyville I, D-St Louis, D-Baltimore, D-Washington, G-Bristol and G-Azakouli. This variant haemoglobin is found in 0.044% of African Caribbeans [33]. It occurs in a variety of other

ethnic groups including African Americans, Algerians, Italians (northern Italy and Sardinia), Chinese and Melanesians. Interestingly, it can result from two different mutations: AAC \rightarrow AAG on a chromosome with the $-\alpha^{3.7}$ deletion and AAC \rightarrow AAA in the $\alpha 2$ gene on a chromosome with the normal complement of two α genes. The former is found in African Caribbeans and African Americans and the latter in Italians.

Haemoglobin G-Philadelphia trait

Haemoglobin G-Philadelphia trait is of no clinical significance. In about 80% of cases this mutated gene occurs on an $-\alpha^{3.7}$ chromosome whereas the other 20% of cases have $\alpha^G\alpha$.

Clinical features

There are no clinical features.

Laboratory features

Blood count

In those with the genotype in $-\alpha^G/\alpha\alpha$ there may be mild anaemia or a normal Hb. In one study the MCV in adults with this genotype was 71–80 fl and the MCH 22.7–26.8 pg [107]. In those who also have α^+ thalassaemia in *trans* the microcytosis is more marked.

Blood film

The blood film is normal.

Other investigations

The variant haemoglobin comprises 20–25%, 30–35% or 45–48% of total haemoglobin with the trimodal distribution likely to indicate groups of individuals with one α^G gene and the number of normal α genes being three, two or one.

On cellulose acetate electrophoresis and HPLC, haemoglobin G-Philadelphia, being an α chain variant, is associated with a variant

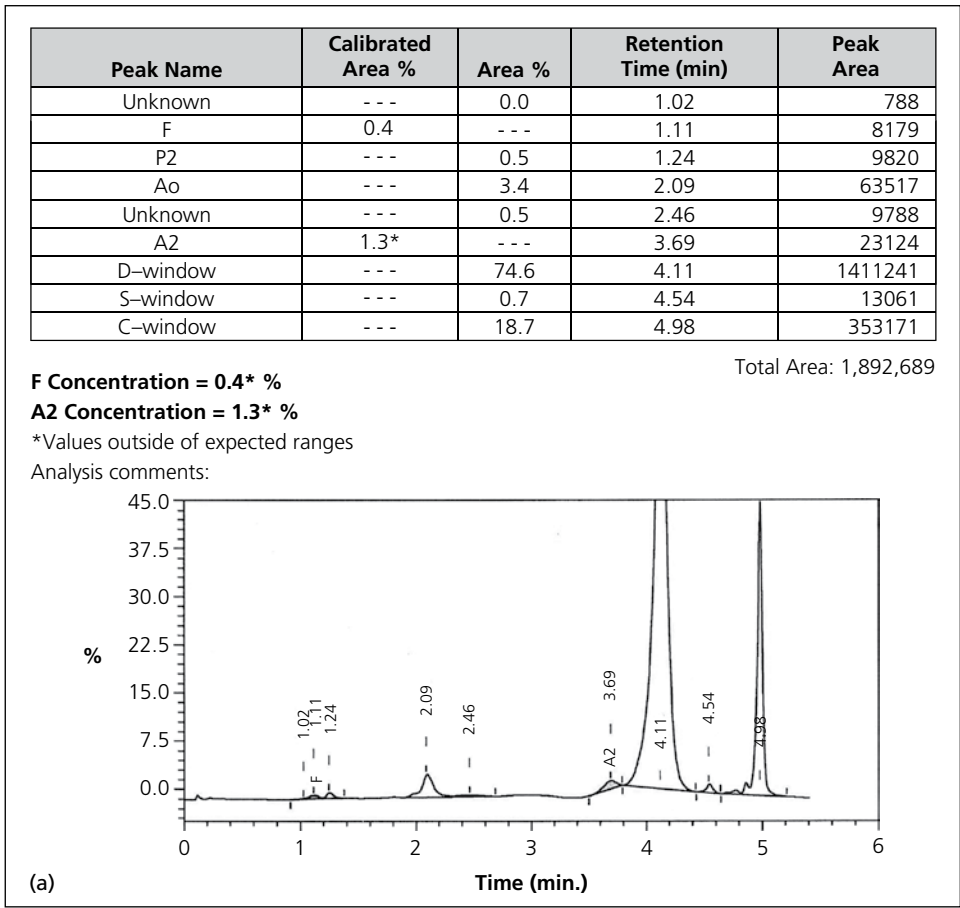


Fig. 5.40 Investigations of a patient who is homozygous for haemoglobin D-Punjab and heterozygous for the α chain variant, haemoglobin Q-India: (a) HPLC (Bio-Rad Variant II) showing major peaks in the D window and the C window (a hybrid haemoglobin with a D-Punjab β chain and a Q-India α chain); the minor peaks are post-translationally modified variant haemoglobins;

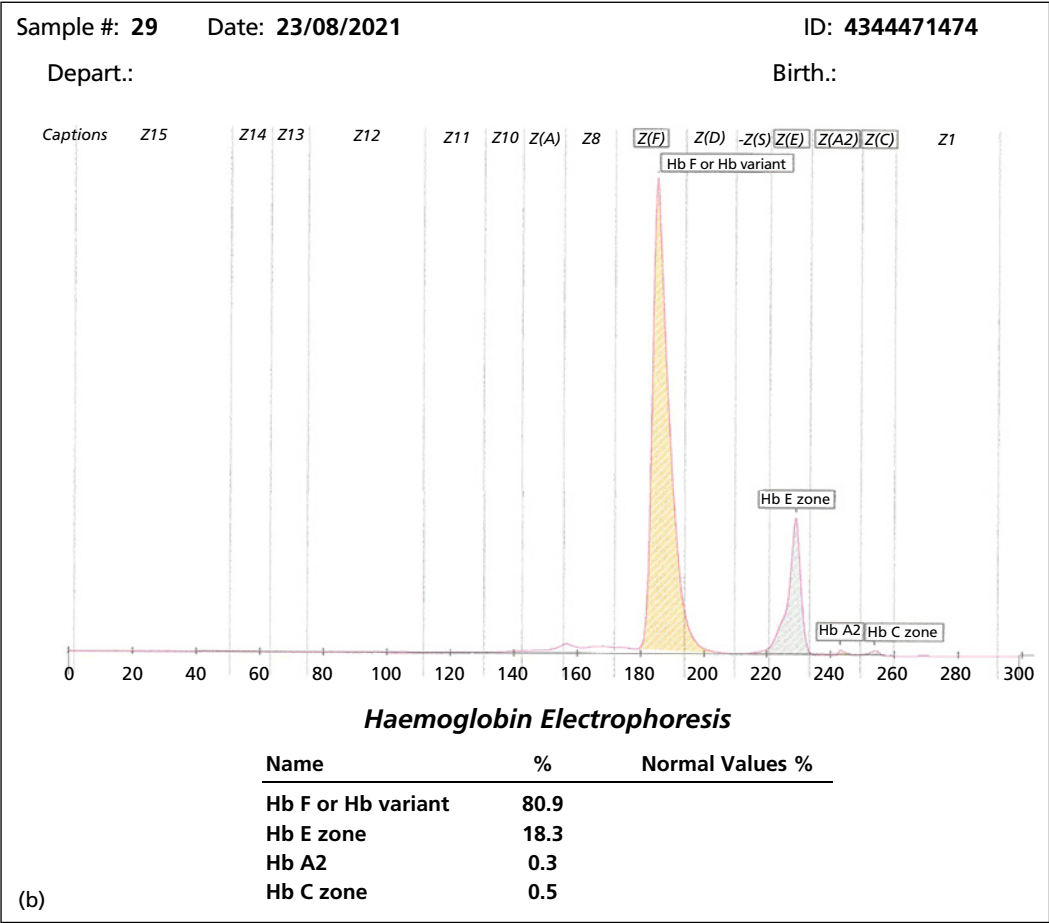


Fig. 5.40 Continued. (b) capillary electrophoresis (Sebia Capillarys) showing peaks in the F zone and the E zone (D-Punjab and the hybrid haemoglobin).

haemoglobin A₂, producing a split haemoglobin A₂ band or peak. Similarly, at birth there is a variant haemoglobin F. The A₂ variant, haemoglobin G₂, has been reported to be present in a lesser amount than haemoglobin A₂, with the total A₂ plus G₂ percentage being normal or slightly elevated [107].

Haemoglobin G-Philadelphia can be distinguished from haemoglobin D-Punjab by isoelectric focusing and HPLC (Fig. 5.41). On capillary electrophoresis, G-Philadelphia appears in the same zone as D-Punjab and Lepore but the lower percentage and the presence of haemoglobin G₂ indicate that it is an α chain variant and permit distinction from D-Punjab (Fig. 5.42).

Diagnosis

The diagnosis requires identification of haemoglobin G-Philadelphia and haemoglobin A by two reliable independent techniques. It can be difficult to detect the split A₂/G₂ band on electrophoresis so that diagnosis by a combination of cellulose acetate alkaline electrophoresis and agarose gel acid electrophoresis is not very reliable. Isoelectric focusing, HPLC and capillary electrophoresis give more dependable information. DNA analysis, usually involving sequencing of the α globin genes, can be used to identify or confirm the diagnosis of haemoglobin G-Philadelphia.

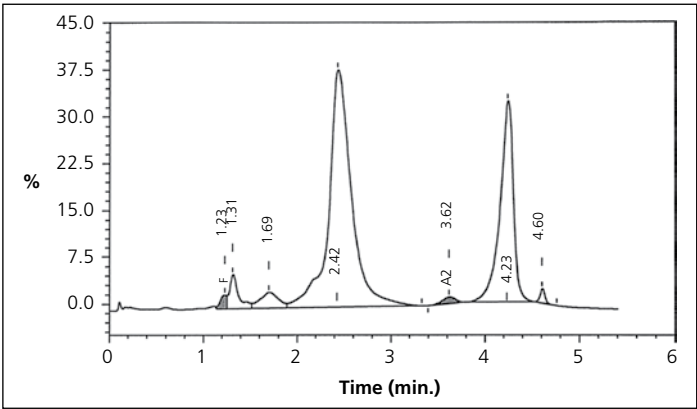


Fig. 5.41 HPLC chromatogram (Bio-Rad Variant II) from a patient with haemoglobin G-Philadelphia heterozygosity; haemoglobin G-Philadelphia appears in the D window and was 30% with a retention time of 4.23 minutes; note that the G₂ fraction, which helps to identify this as an α chain variant, appears in the S window; peaks, from left to right, are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks) and haemoglobins A₀, A₂ (shaded), G and G₂.

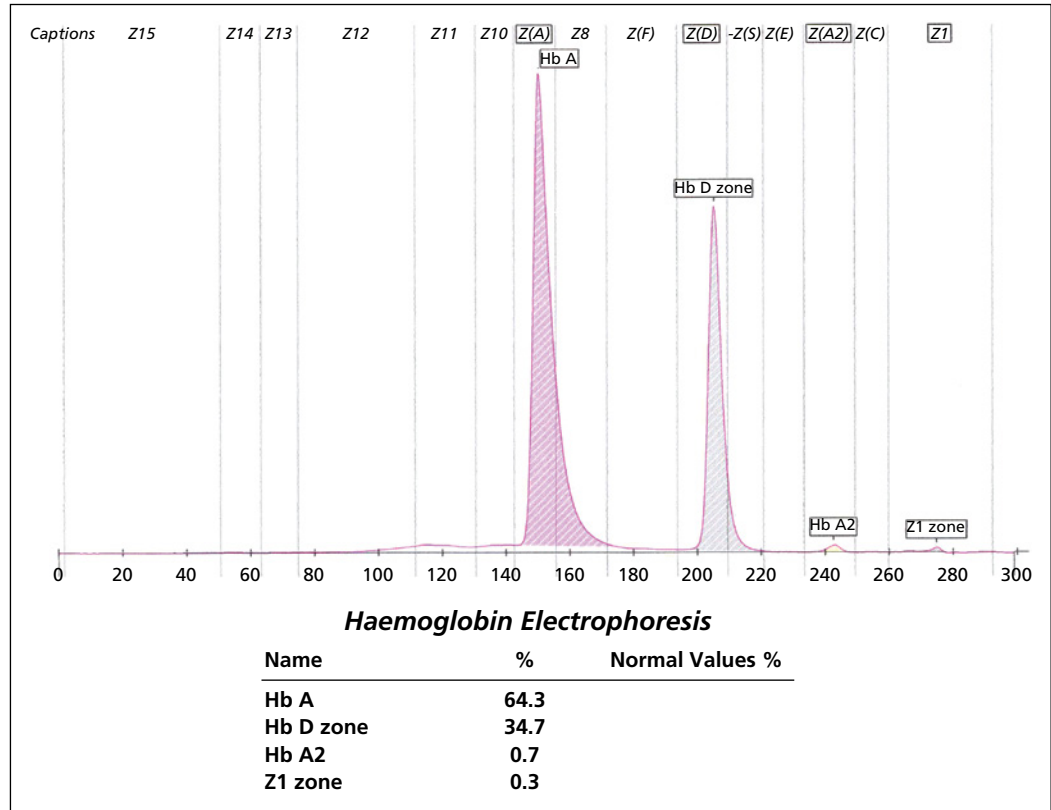


Fig. 5.42 Capillary electrophoresis (Sebia Capillarys 3) in haemoglobin G-Philadelphia heterozygosity. The peaks from left to right are haemoglobin A, haemoglobin G-Philadelphia, haemoglobin A₂ and haemoglobin G₂.

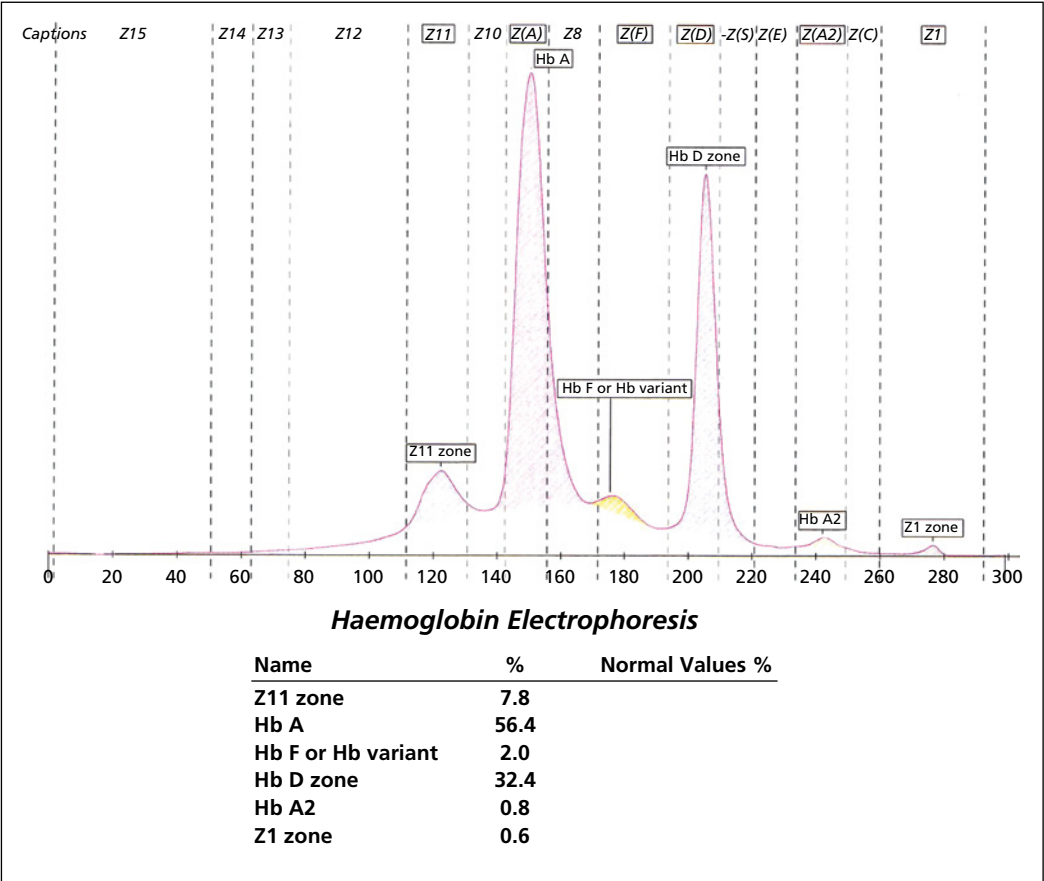


Fig. 5.42 Continued.

Haemoglobin G-Philadelphia homozygosity and coinheritance of haemoglobin G-Philadelphia and other variant haemoglobins or thalassaemias

Homozygosity for $-\alpha^G$ has been reported. There is marked hypochromia and microcytosis.

Compound heterozygosity for $-\alpha^G$ and α^0 thalassaemia leads to haemoglobin H disease with haemoglobin G-Philadelphia but no haemoglobin A.

Individuals who are heterozygous for both haemoglobin G-Philadelphia and haemoglobin D-Punjab may have a normal Hb or mild anaemia [108]. The blood film and reticulocyte count are normal. Haemoglobin electrophoresis at alkaline pH shows 30–40% haemoglobin A, approximately 45% haemoglobin with the mobility of S (representing haemoglobin

D-Punjab and haemoglobin G-Philadelphia), approximately 15% haemoglobin with the mobility of C/E/A₂ (representing haemoglobin A₂ and the hybrid G-Philadelphia/D-Punjab haemoglobin) and about 2% of haemoglobin G₂. Haemoglobin electrophoresis at acid pH is normal since both variant haemoglobins move with haemoglobin A.

Coinheritance of haemoglobin G-Philadelphia with haemoglobin S heterozygosity and homozygosity has been discussed on pages 222 and 249, and with haemoglobin C and haemoglobin S plus C on pages 313 and 260.

Haemoglobin O-Arab

Haemoglobin O-Arab (also reported as haemoglobin Egypt) is a β chain variant, $\alpha_2\beta_2^{122\text{Glu}\rightarrow\text{Lys}}$. It has been found in a great variety of ethnic

groups but is not common in any of them. Despite its name, it is actually quite uncommon among Arabs. It appears to be of African rather than Arab origin, with a distribution similar to that of the Ottoman empire [109]. It has been suggested that it entered the Turkish domain with Sudanese contingents of the Turkish army or, alternatively, that it arose among the Greek Pomaks, who have the highest prevalence known [110]. In addition to its occurrence among Arabs (in Israel, Yemen, Egypt, Saudi Arabia), it has been reported in Greeks, in Italians, in eastern Europe (Bulgaria, the Balkans, Hungary, Albania), in gipsies and among Africans (Kenyans, Sudanese, Moroccans, Tunisians) and those of African descent. In a survey in Jamaica the prevalence was 0.016% [33]. Its main importance is because of possible interaction with haemoglobin S (see page 268) and β thalassaemia.

Haemoglobin O-Arab trait

Haemoglobin O-Arab trait is of genetic but no other clinical significance.

Clinical features

Haemoglobin O-Arab trait causes no clinical abnormality.

Laboratory features

Blood count

The Hb is normal. The MCV may be normal or borderline low.

Blood film

The blood films can show slight anisocytosis, poikilocytosis, hypochromia and a few target cells [23].

Other investigations

The percentage of dense red cells is increased [9]. Haemoglobin O-Arab is 38–43% of total haemoglobin. It has a mobility similar to haemoglobin

C at alkaline pH (Fig. 5.43). On citrate agar at acid pH it moves close to S but slightly ahead whereas on agarose gel at acid pH it moves close to, but slightly behind, haemoglobin S (Fig. 5.44). On HPLC, haemoglobin O-Arab elutes in or near the C window but has a retention time between those of haemoglobins S and C (Fig. 5.45) (see also Fig. 2.19o). In addition, there is a minor peak in the haemoglobin D window [111] and another peak also representing

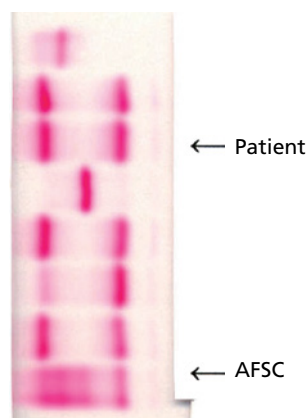


Fig. 5.43 Cellulose acetate electrophoresis at alkaline pH in a patient with haemoglobin O-Arab heterozygosity; haemoglobin O-Arab has the same mobility as haemoglobins C and E but is present as a higher percentage than haemoglobin E; AFSC indicates a control sample containing haemoglobins A, F, S and C.

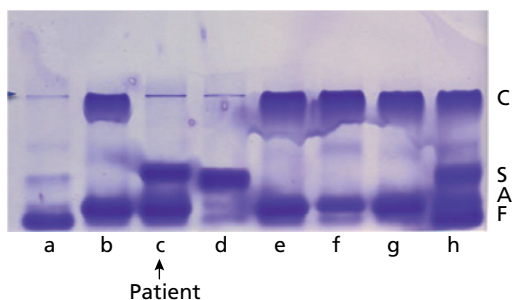


Fig. 5.44 Agarose gel electrophoresis at acid pH in a patient with haemoglobin O-Arab heterozygosity: lanes show, from left to right: (a) F plus faint S; (b) A+C; (c) A plus O-Arab; (d) faint F and A plus S; (e, f and g) A plus C; (h) A, F, S and C (control sample); note that the haemoglobin O-Arab is slightly slower than haemoglobin S, i.e. slightly closer to C.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.02	1986
F	0.6	---	1.10	14232
Unknown	---	0.5	1.27	13224
P2	---	2.2	1.36	56592
P3	---	2.6	1.77	67630
Ao	---	52.0	2.48	1328484
A2	2.4	---	3.68	62888
D-window	---	1.2	4.01	31742
S-window	---	0.4	4.60	11198
Unknown	---	37.9	4.87	967547

Total Area: 2,555,522

F Concentration = 0.6 %

A2 Concentration = 2.4 %

Analysis comments:

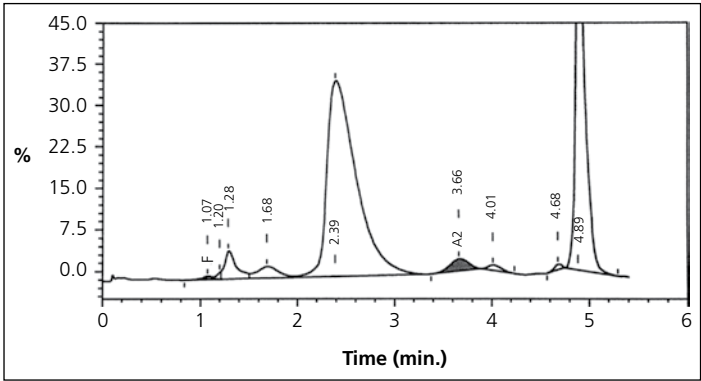
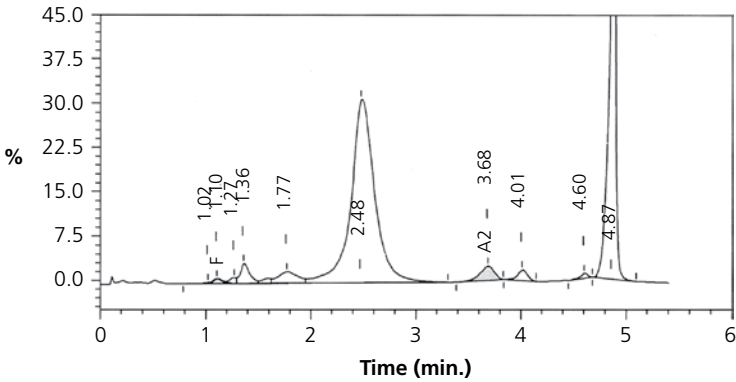


Fig. 5.45 At HPLC chromatogram (Bio-Rad Variant II) in a patient with haemoglobin O-Arab heterozygosity; the major peaks, from left to right, are post-translationally modified haemoglobin A (two peaks), haemoglobins A₀ and A₂ (shaded), two small peaks representing glycosylated O-Arab and other post-translationally modified O-Arab, and O-Arab.

post-translationally modified O-Arab close to the main peak. On capillary electrophoresis, the O-Arab peak appears in the haemoglobin A₂ zone (Fig. 5.46). The relatively low percentage may result, as with haemoglobins S and C, from the positive charge of the $\beta^{\text{O-Arab}}$ chain. Individuals with coexisting α thalassaemia trait have a somewhat lower percentage of the variant haemoglobin.

Diagnosis

If the primary diagnostic method is cellulose acetate electrophoresis at alkaline pH, the differential diagnosis is with haemoglobins C, E and C-Harlem. Physicochemical characteristics are very similar to those of haemoglobin C-Harlem but in the simple heterozygotes the distinction can be made

easily because C-Harlem has a positive sickle solubility test. If the primary diagnostic method is HPLC, haemoglobin C-Harlem must again be considered but it is rare in comparison with O-Arab.

Haemoglobin O-Arab disease

Homozygosity for haemoglobin O-Arab has been reported in Bulgaria, the Balkans (a Roma family), Morocco, Tunisia, the Sudan and Kenya [23, 109, 112–114].

Clinical features

Homozygotes may be asymptomatic with compensated haemolysis or there may be recurrent jaundice and anaemia. The spleen may be enlarged.

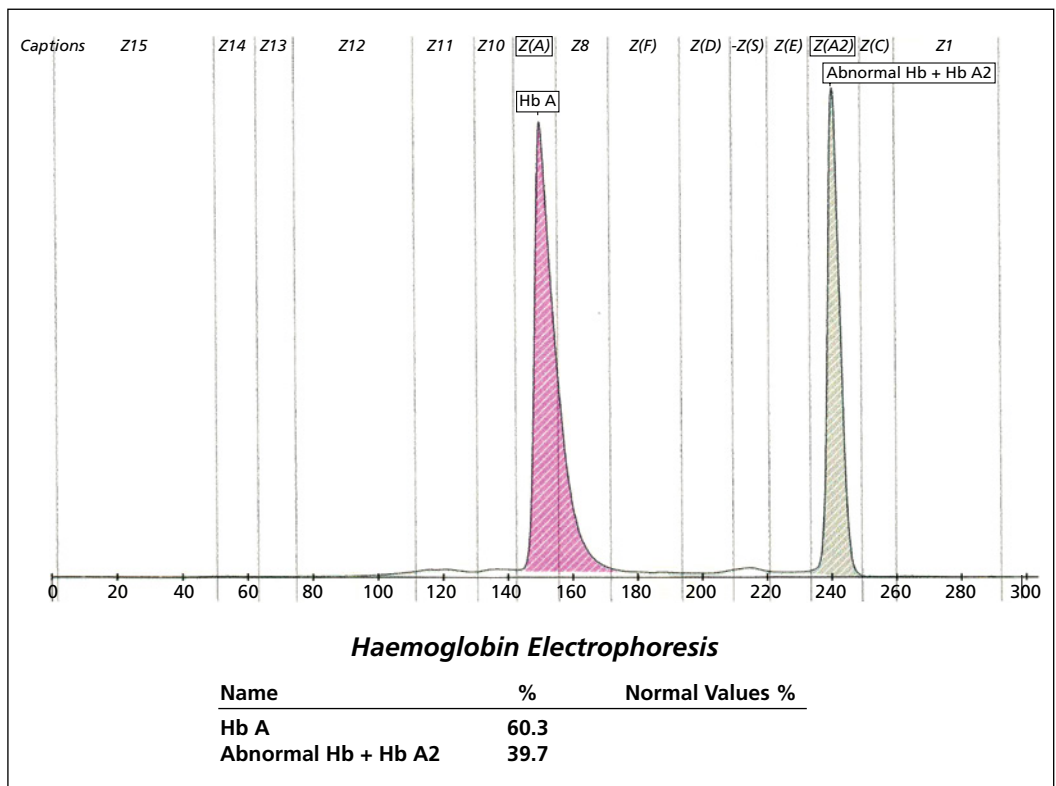


Fig. 5.46 Capillary electrophoresis (Sebia Capillarys) from a patient with haemoglobin O-Arab heterozygosity showing haemoglobin O-Arab in the haemoglobin A₂ zone.

Laboratory features

Blood count

The Hb can be normal or reduced and the MCV is reduced. The reticulocyte count is mildly increased.

Blood film

The blood film shows numerous target cells and sometimes NRBC.

Other investigations

Haemoglobin O-Arab comprises almost all the haemoglobin with a small amount of haemoglobin A₂. Haemoglobin F may be increased, at least in children [115]. The percentage of dense cells is increased, the abnormality being comparable to that seen in homozygosity for haemoglobin C [9].

Haemoglobin O-Arab/ β^0 thalassaemia

Haemoglobin O-Arab/ β^0 thalassaemia has been described in Bulgaria, Hungary and Italy and haemoglobin O-Arab/ β^+ thalassaemia in Saudi Arabia, Turkey and in an African Caribbean patient [23, 25].

Clinical features

This compound heterozygous state causes slight to moderate anaemia with jaundice and splenomegaly. The haemolytic anaemia may be episodic, being precipitated by intercurrent infection.

Laboratory features

Blood count

The reported Hb has ranged from 60 g/l to normal. The MCV is reduced and the reticulocyte count is mildly increased.

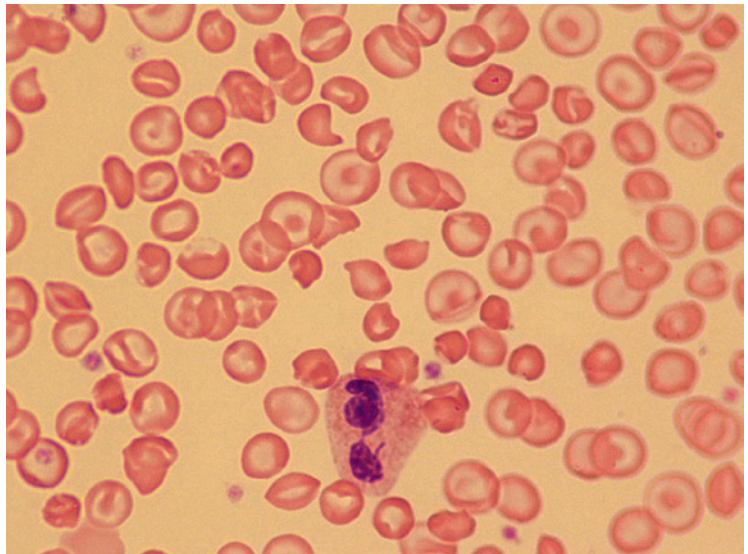
Blood film

The blood film (Fig. 5.47) shows anisocytosis, poikilocytosis, hypochromia, microcytosis and target cells, which may be infrequent or numerous.

Other investigations

Haemoglobins present are O and A₂ with or without haemoglobin A. Haemoglobin F may be mildly increased.

Fig. 5.47 Blood film of a patient with compound heterozygosity for haemoglobin O-Arab and β^0 thalassaemia showing target cells and irregularly contracted cells. The patient was of Turkish Cypriot, Egyptian and Jamaican ancestry. Red cells indices were RBC 6.78×10^9 /l, Hb 121 g/l, MCV 55.4 fl, MCH 17.8 pg and MCHC 320 g/l. MGX $\times 100$.



Coinheritance of haemoglobin O-Arab and other variant haemoglobins or thalassaemias

Coinheritance of haemoglobin S and haemoglobin O-Arab causes sickle cell disease. This condition has been described on page 268.

Unstable haemoglobins

The term 'unstable haemoglobin' is best restricted to those variant haemoglobins that cause clinically significant haemolysis. Other haemoglobins, for example haemoglobin E, are unstable *in vitro* but this probably does not contribute significantly to the associated clinical features.

Both α and β chain variant haemoglobins can be unstable. A small number of γ chain variants have been described that lead to haemoglobin instability and haemolysis in neonates, including haemoglobin F-Poole [116], haemoglobin F-Bonheiden [117] and haemoglobin Wareham [118]. In addition, there are two γ chain variants that are methaemoglobins and also unstable, haemoglobin F-M-Fort Ripley and haemoglobin F-M-Osaka, which cause neonatal pseudocyanosis [119]. Occasionally, the fetal form of an α chain variant is more unstable than the adult form; this is the case with haemoglobin Hasharon, with $\alpha_2^{\text{Hasharon}}\gamma_2$ being more unstable than $\alpha_2^{\text{Hasharon}}\beta_2$ [120] and being associated with haemolysis in neonates. Occasionally, a β chain variant is associated with neonatal jaundice [121]. δ chain variants can also be unstable but because of their low concentration, this is of no clinical significance. Several patients have been described with two unstable haemoglobins, together with haemoglobin A or a β^0 -thalassaemia determinant. This results not from three β genes in the genome but from post-translational modification of an unstable haemoglobin. Both haemoglobin Sydney and haemoglobin Atlanta can be modified in this manner, producing haemoglobin Sydney-Coventry and haemoglobin Atlanta-Coventry respectively. Haemoglobin instability can result from an unstable globin chain or an unstable haemoglobin molecule. The causative primary

abnormality can affect the secondary structure (α helix and intervening turns), the tertiary three-dimensional structure of the monomer or the quaternary structure (relationship between monomers).

Haemoglobin instability may be consequent on:

- an abnormality of the haem pocket so that haem is not firmly bound and water can enter the normally hydrophobic haem pocket; haem depleted dimers and tetramers are present;
- interference with the α helical structure, often because an amino acid is replaced by the imino acid, proline, or interference with the interhelical bends (abnormality of secondary structure);
- replacement of an internal non-polar amino acid with a polar amino acid, which has to be oriented outwards and thus disrupts the molecule (interference with tertiary structure);
- interference of the binding of α and β subunits to each other, specifically impairment of the $\alpha_1\beta_1$ dimeric bonds (interference with quaternary structure); this can result in dissociation into monomers, which favours methaemoglobin formation;
- elongation of the β chain;
- probably interference of binding of α chain to α haemoglobin stabilising protein.

The first unstable haemoglobins identified were two β chain variants, haemoglobin Zurich and haemoglobin Köln, both identified in the early 1960s. Subsequently, a case of 'congenital non-spherocytic haemolytic anaemia' reported in the 1950s was found to be attributable to haemoglobin Bristol. Since these early reports, more than 135 unstable haemoglobins have been described, of which haemoglobin Köln appears to be the most common. Many more unstable β variants have been reported than unstable α variants, perhaps because α variants, being a lower proportion of total haemoglobin, are more likely to go unrecognised.

Mutations leading to an unstable haemoglobin can be:

- a point mutation leading to replacement of one amino acid by another or replacement of an amino acid by the imino acid, proline [23];
- a point mutation followed by post-translational modification of the haemoglobin

encoded; leucine in an abnormal haem pocket is modified to hydroxyleucine [122, 123];

- deletions of one to eight codons leading to deletion of a small number of amino acids; for example, haemoglobin Gun Hill has a β chain that lacks five amino acids including the haem-binding site [124] and haemoglobin J-Biakra (unstable only *in vitro*) has an α chain that lacks eight amino acids [125];
- tandem duplication of codons leading to duplication of a small number of amino acids; for example, haemoglobin Fairfax has five extra amino acids [126], the same amino acids that are deleted in haemoglobin Gun Hill;
- frameshift or STOP codon mutations leading to synthesis of an elongated β chain.

Of these various mechanisms, by far the most common is a single amino acid substitution.

Unstable haemoglobins show a greater or lesser tendency to Heinz body formation. Heinz bodies are composed of hemichromes, which are derivatives of ferric haemoglobin (methaemoglobin) in which the haem has been lost from the haem pocket and has bound elsewhere to denatured globin [127]. Heinz bodies bind to the inner surface of the red cell membrane, probably by hydrophobic interactions rather than covalent bonds [127]. Binding is preferentially to band 3. The membrane can also be damaged by free haem and free iron leading to lipid peroxidation. The cell becomes less deformable and may be trapped in the spleen; membrane loss and removal of Heinz bodies occur in the spleen. Recognition of unstable haemoglobins causing significant methaemoglobinaemia can be important because of a possible response to riboflavin [128].

Most unstable haemoglobins cause haematological abnormalities in heterozygotes and homozygosity has not been described. One exception to this generalisation is haemoglobin Bushwick, which is only slightly unstable; it causes no significant abnormality in heterozygotes but caused significant haemolytic anaemia in one described homozygote [129]. Another exception is haemoglobin Taybe, an α chain variant, which causes mild anaemia in heterozygotes and a more severe haemolytic anaemia with hypochromia and microcytosis in homozygotes or compound heterozygotes with α thalassaemia

[130]; homozygous triplets with hydrops fetalis have been described [131]. Unstable haemoglobins have been described in a great variety of different ethnic groups and sometimes an identical mutation has been found in a small number of individuals in very different parts of the world, indicating that independent mutations have occurred. A significant proportion of unstable haemoglobins, probably about a third, are new mutations, both parents being normal.

Unstable haemoglobins may, in addition to being unstable, show either increased or decreased oxygen affinity. Interaction with 2,3-DPG can be impaired. They can also be particularly prone to oxidation to methaemoglobin. Haemoglobin Zurich has an unusual abnormality of the haem pocket, which is associated with an increased affinity for carbon monoxide and an increase in carboxyhaemoglobin; paradoxically, this reduces instability so that Heinz body haemolytic anaemia is less likely in smokers with haemoglobin Zurich than in non-smokers [1, 127]. Some unstable haemoglobins are synthesised at a reduced rate. For example, reticulocytes of a heterozygote for haemoglobin Köln synthesised haemoglobins A and Köln in a ratio of 80:20 [132].

The proportion of abnormal haemoglobin for unstable β chain variants is very variable, ranging from 35–40% in the case of haemoglobin Hammersmith to 10–15% in the case of haemoglobin Köln to almost undetectable in the case of very unstable haemoglobins such as haemoglobin Cagliari. The proportion of an unstable β chain variant is determined by:

- the rate of synthesis of the variant chain;
- the rate of breakdown of the β^{VARIANT} chain before association with α chain can occur;
- the rate of association of α and β^{VARIANT} chains in comparison with the rate of association of α chains and normal β^{A} chains;
- the rate of breakdown of $\alpha\beta^{\text{VARIANT}}$ dimers before assembly of dimers to tetramers can occur;
- the rate of denaturation of unstable haemoglobin (with consequent removal as Heinz bodies).

In the case of unstable α chain variants the proportion of variant haemoglobin is usually less

than 15% but may be 1–2% or even less. The explanation for the variable proportion of the variant haemoglobin is as for the β chain variants.

In some instances the proportion of an unstable haemoglobin is also affected by the fact that the mutation occurs in *cis* to an α thalassaemia determinant (Hb Suan-Dok, Haemoglobin Petah Tikva) or in *trans* to a β thalassaemia determinant (haemoglobin Leiden) [127].

Depending on the degree of instability of a variant globin chain or of a variant haemoglobin, various degrees of abnormality are possible: (i) a very unstable α or β chain is destroyed so rapidly that no variant globin chain or haemoglobin is detectable and the phenotype is that of dominant thalassaemia; (ii) an unstable globin binds haem but cannot bind to other globin chains so that it precipitates as Heinz bodies in erythroid precursors, leading to dyserythropoietic and ineffective erythropoiesis, as in dominant β thalassaemia intermedia resulting from heterozygosity for a hyperunstable β chain; (iii) a lesser degree of instability permits a variant haemoglobin to be synthesised leading to a haemolytic anaemia and the clinical features recognised in association with an unstable haemoglobin. Highly unstable α chain variants are usually clinically silent but if they interact with an α thalassaemia determinant the phenotype may either be that of haemoglobin H disease or a haemolytic anaemia or may simulate β thalassaemia intermedia; in the latter instance there is no haemoglobin H detectable, there is dyserythropoiesis and globin chain synthesis studies show a paradoxically increased α : β ratio [133]. A phenotype with features of both thalassaemia and a Heinz body haemolytic anaemia can be produced either by a reduced rate of synthesis of an unstable variant or by marked instability leading to destruction of the variant globin chain or of $\alpha\beta$ dimers before assembly of haemoglobin tetramers can occur.

Very unstable haemoglobins may not only have a phenotype that simulates β thalassaemia major but may have marked dyserythropoiesis so that confusion with a congenital dyserythropoietic anaemia is possible [134]. Patients with a very unstable haemoglobin are sometimes treated by

splenectomy. Although this can lead to a reduction in haemolysis, there may also be an increase in the platelet count, leading to thrombotic complications, and it is difficult to predict whether the benefits of splenectomy will outweigh the risks. They are also sometimes treated with hydroxycarbamide, leading to a beneficial increase in haemoglobin F percentage and a fall in the percentage of the unstable variant.

Clinical features

An unstable haemoglobin can cause severe, moderate or mild anaemia. Depending on the severity of the abnormality and the chain that is abnormal, presentation may be in infancy, childhood or adult life. One α chain variant, haemoglobin Hasharon, causes significant haemolysis in neonates but not in adult life, since $\alpha_2^{\text{Hasharon}}\gamma_2$ is more unstable than $\alpha_2^{\text{Hasharon}}\beta_2$ [1]. There may be intermittent or constant jaundice and passage of dark brown or almost black urine, the latter attributable to excretion of dipyrroles, which are abnormal breakdown products of haem. The incidence of gallstones is increased and they can occur in children [135]. Leg ulcers can occur. The spleen may be enlarged and hypersplenism sometimes occurs. When haemolysis is severe and chronic, pulmonary hypertension can occur. Rarely, *moya moya* has been described [136]. Anaemia may be intermittent or may intermittently worsen. Such deterioration is often attributable to intercurrent febrile infections or exposure to oxidant drugs. In the case of haemoglobin Zurich, haemolysis may occur only on exposure to oxidants, and smokers, for the reason described on page 347, exhibit less haemolysis than non-smokers. Acute deterioration caused by folic acid deficiency and red cell aplasia due to parvovirus B19 infection have also been recognised. When an unstable haemoglobin is abnormally prone to oxidation to methaemoglobin the patient may be cyanosed. Low oxygen affinity of an unstable haemoglobin can also cause cyanosis.

Some variant haemoglobins are only slightly unstable so that although *in vitro* tests for instability are positive, there is usually no associated clinical abnormality.

Laboratory features

Blood count

The Hb may be normal, except during episodic haemolysis, or may be mildly, moderately or severely reduced. On average, the Hb is higher when the unstable haemoglobin also has increased oxygen affinity. The MCV can be elevated and the MCH and MCHC reduced. The reduction of MCH and MCHC may be attributable to loss of haem or removal of Heinz bodies by the spleen. Loss of haem from the haem pocket can lead to a low MCHC despite normocytic cells in the blood film since the staining of the red cells in a blood film is attributable to the globin content whereas the chemical measurement of haemoglobin is dependent on the haem molecule [124]. The reticulocyte count may be elevated constantly or intermittently. The degree of elevation of the reticulocyte count is not necessarily proportional to the reduction of the Hb because of the effect of altered oxygen affinity. If an unstable haemoglobin has a high oxygen affinity the reticulocyte count will be higher, in relation to the Hb, than if there is an unstable haemoglobin with a low oxygen affinity. There can be thrombocytopenia, which is sometimes disproportionate to the degree of splenomegaly and not readily explicable.

Occasionally, when a mildly unstable haemoglobin has increased oxygen affinity, there is polycythaemia rather than anaemia. Polycythaemia has also occasionally been observed to develop following splenectomy.

During acute increases in the rate of haemolysis there is a fall in the Hb and a rise in the reticulocyte count. The white cell count and the neutrophil count usually also rise.

Blood film

The blood film (Fig. 5.48) can show anaemia, macrocytosis, polychromatic macrocytes, mild hypochromia, basophilic stippling and the presence of irregularly contracted cells and keratocytes ('bite cells'). Sometimes there is hypochromia and microcytosis [130]. Although it is often considered that Heinz bodies cannot be detected on a Romanowsky-stained film, it

has been pointed out that they can in fact be identified in patients who have required splenectomy because of the presence of an unstable haemoglobin (and also in other patients with severe oxidant-induced haemolytic anaemia) [23].

During haemolytic crises there is an increase in polychromasia and in the number of irregularly contracted cells and keratocytes. Functional hyposplenism, consequent on reticuloendothelial overload, can lead to the appearance of Howell-Jolly bodies.

Other investigations

Haemoglobin electrophoresis may be abnormal (Fig. 5.49) but is basically normal in more than half of the reported unstable haemoglobins, specifically in those with no change in charge [1]. If the variant haemoglobin has a normal electrophoretic mobility there may nevertheless be a tail behind the main haemoglobin band representing either denatured haemoglobin or haemoglobin with a variable degree of haem depletion [137]; such bands are more likely if the specimen is not fresh. Disappearance of an abnormal band when haemin is added demonstrates that it results from haem depletion. In the case of unstable β chain variants, free α chains may form a discrete band near the origin, just behind haemoglobin A₂. Occasionally, two abnormal bands have been reported, for example in the case of a heterozygote for haemoglobin Rush, apparently representing migration of asymmetric hybrids ($\alpha_2\beta\beta^{\text{Rush}}$) as well as haemoglobin A and haemoglobin Rush [1].

Some unstable haemoglobins appear as a discrete peak on HPLC; in others there is a minor component apparent, representing degraded haemoglobin (Fig. 5.50a). Capillary electrophoresis may similarly show small peaks representing degraded variant haemoglobin (Fig. 5.50b). The shape and number of minor peaks vary according to the age of the sample. Sometimes standard electrophoretic and HPLC techniques all give normal results and DNA analysis or mass spectrometry is required for demonstration of the genetic change or variant haemoglobin.

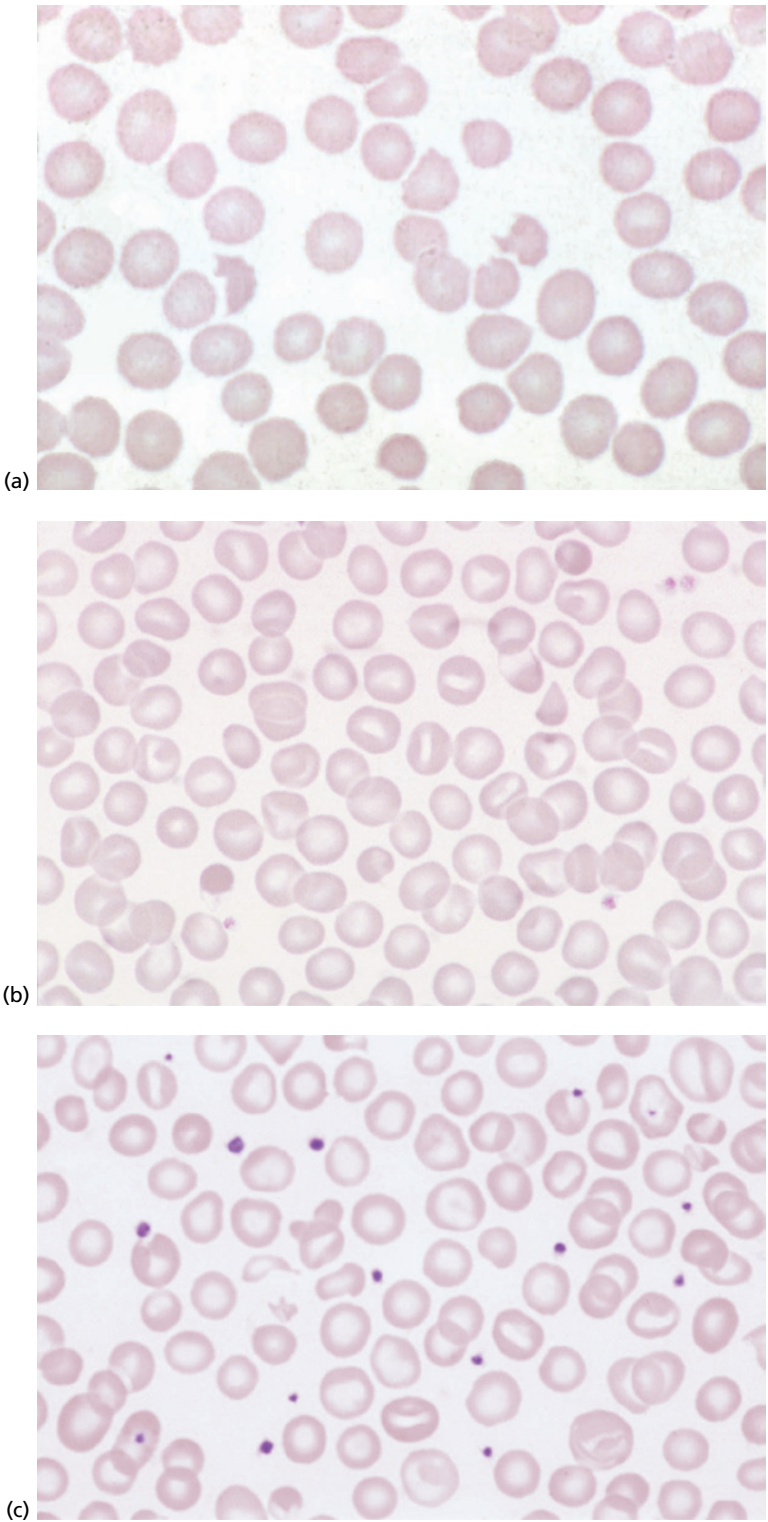


Fig. 5.48 Blood films of three patients with unstable haemoglobins showing: (a) irregularly contracted cells, macrocytosis and thrombocytopenia in a patient with haemoglobin Köln; (b) several stomatocytes and several irregularly contracted cells in a patient with haemoglobin Siriraj; and (c) hypochromia and poikilocytosis in a patient with haemoglobin St Mary's. MGG $\times 100$.

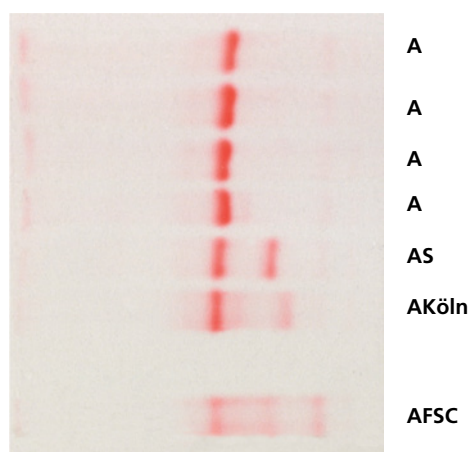


Fig. 5.49 Haemoglobin electrophoresis on cellulose acetate at alkaline pH in a patient with haemoglobin Köln; a faint band representing denatured haemoglobin Köln is apparent behind haemoglobin A and between S and C positions; AFSC, control sample.

When the unstable haemoglobin is a β chain variant the concentration of haemoglobin A_2 may be increased, as a consequence of selective denaturation and removal of the unstable variant. Haemoglobin F concentration can be moderately increased (e.g. to 10–12%). Methaemoglobin may be present in fresh blood or, more often, appears abnormally rapidly as the specimen ages.

Heat and usually isopropanol tests for haemoglobin instability are positive. The instability of some haemoglobins can also be demonstrated by their response to mechanical shaking. The heat test is more sensitive than the isopropanol test so will detect variant haemoglobins showing a lesser degree of instability. For example, the instability of haemoglobin Olmsted is detected only by a heat test [138]. For this reason it has been suggested that both tests be employed. Interestingly, the colour of the precipitate varies, depending on the tendency of the unstable haemoglobin to lose haem. Thus haemoglobin Hammersmith gives a red-brown precipitate representing haemoglobin Hammersmith with most of its haem groups still attached whereas haemoglobin Santa Ana loses haem groups so readily that the precipitate is almost white [23]. Paradoxically, when a globin

chain or haemoglobin is very unstable heat and isopropanol tests may be negative [134, 139].

A Heinz body test may be positive or may become positive on incubation of the blood at 37 °C for 24 hours. During acute haemolytic episodes, including those induced by drugs, a previously negative Heinz body test may become positive. This is both because of an increased rate of formation of Heinz bodies and because reticuloendothelial overload means that, once formed, Heinz bodies are not being pitted from red cells by hepatic and splenic macrophages. If a splenectomy has been necessary, a Heinz body test is likely to be positive, with Heinz bodies sometimes being present in almost every red cell.

Oxygen affinity may be increased (e.g. haemoglobin Köln) or reduced (e.g. haemoglobin Hammersmith). Of the reported unstable haemoglobins, oxygen affinity has been found to be reduced in 30%, normal in 20% and increased in 50% [1]. Since these conditions are generally heterozygous, the oxygen dissociation curve is generally biphasic, representing the mixture of normal and variant haemoglobins. Red cell life span is reduced but measurements may be inaccurate for the following reasons: ^{51}Cr may bind preferentially to the unstable haemoglobin; ^{51}Cr may lead to denaturation of the unstable haemoglobin; loss of ^{51}Cr may indicate removal of a Heinz body by the spleen rather than death of a red cell [23].

Bone marrow examination (Fig. 5.51) may show erythroid hyperplasia and dyserythropoietic features such as bi- and multinuclearity and nuclear irregularity or lobulation [140]; ring sideroblasts are sometimes present.

There may be an increase in bilirubin concentration (mainly unconjugated bilirubin) and LDH together with evidence of intravascular haemolysis such as a reduced serum haptoglobin concentration, reduced serum haemopexin and the presence of serum methaemalbumin and urinary haemosiderin.

It should be noted that inaccurate (low) pulse oximeter measurements of oxygen saturation have been reported, as a result of the abnormal absorption spectrum of the variant haemoglobin, with a number of unstable haemoglobins including

haemoglobin Köln, haemoglobin Hammersmith and haemoglobin Cheverly [141, 142].

Diagnosis

Electrophoresis, HPLC or both should be performed. However, since other tests may all give normal results, diagnosis requires a specific test for an unstable haemoglobin followed by DNA analysis or mass spectrometry. A blood film examination is particularly useful. Where available, DNA analysis using a panel of genes is increasingly used early in the investigation of

congenital non-spherocytic haemolytic anaemia, and will detect the presence of globin chain variants predicted to cause haemoglobin instability. Further laboratory analysis, such as a heat stability test, can then be performed to confirm the predicted phenotype.

Coinheritance of an unstable haemoglobin and other variant haemoglobins or thalassaemia

The severity of disease caused by an unstable β chain variant may be reduced by coinheritance

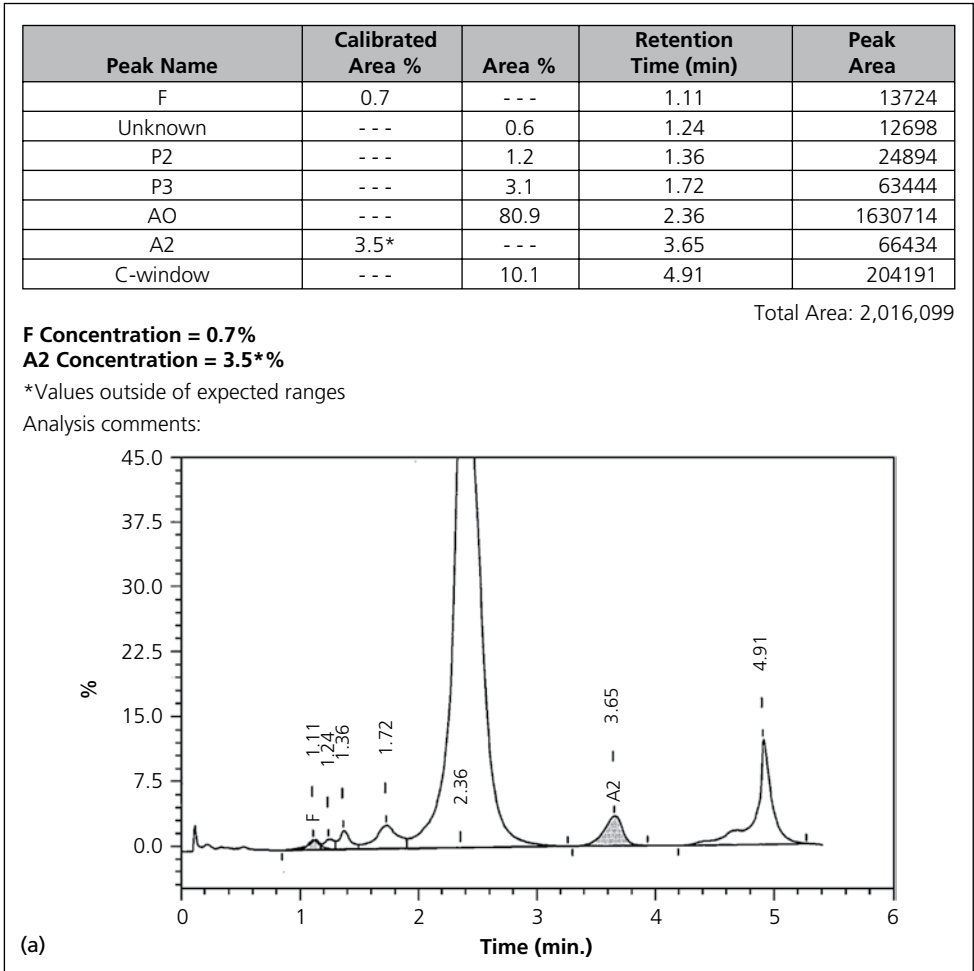


Fig. 5.50 Investigations on a patient who was heterozygous for haemoglobin Köln: (a) HPLC chromatogram (Bio-Rad Variant II) showing minor components that represent denatured haemoglobin Köln (far right); other peaks, from left to right, are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobins A_0 plus unaltered haemoglobin Köln and haemoglobin A_2 (shaded);

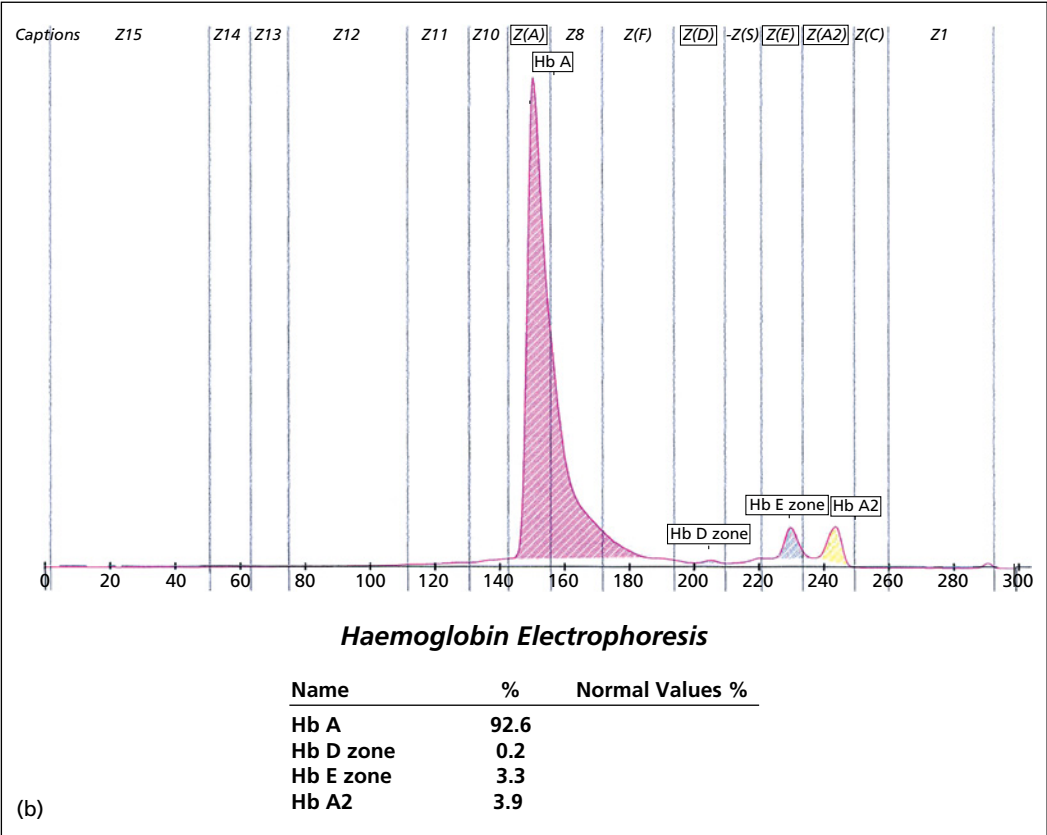


Fig. 5.50 *Continued.* (b) capillary electrophoresis (Sebia Capillars) showing degraded haemoglobin Köln in the haemoglobin E zone; the patient also had a borderline increase in haemoglobin A₂.

of α thalassaemia [143]. Coinheritance of an unstable haemoglobin (e.g. haemoglobin Acharnes, haemoglobin Arta or haemoglobin Lulu Island) with β thalassaemia has been associated with the clinical picture of β thalassaemia intermedia (see Table 3.10). Coinheritance of an unstable haemoglobin and β^0 thalassaemia in *trans* can lead to a severe haemolytic anaemia [144].

Haemoglobin M

The designation haemoglobin M is given to a variety of α , β and γ chain haemoglobin variants that show an increased tendency to oxidation to methaemoglobin, with resultant cyanosis or, more correctly, pseudocyanosis (Fig. 5.52). Methaemoglobin itself is actually dark brown, with the blue colour emerging as the light is filtered through the skin. The abnormal colour of

the patient's skin differs from the purplish-blue of true cyanosis and has been variously described as lavender blue, slate grey or brownish-slate. However, with haemoglobins M-Boston and M-Iwate, which have a low oxygen affinity, there is also an element of true cyanosis [127]. In Japan, terms meaning 'black mouth' or 'black blood' or 'black child' were used for carriers of haemoglobin M-Iwate with the phenotype being recognised as early as the 1800s [145]. The first haemoglobin M was recognised in 1948, a year before the first description of haemoglobin S [146].

The molecular abnormality is usually replacement of a histidine residue in the haem pocket, by tyrosine, so that the iron of the haem molecule is stabilised in the ferric (Fe^{3+}) form. Either the proximal or the distal histidine may be involved (Fig. 5.53). One exception is haemoglobin M-Milwaukee in which the longer side

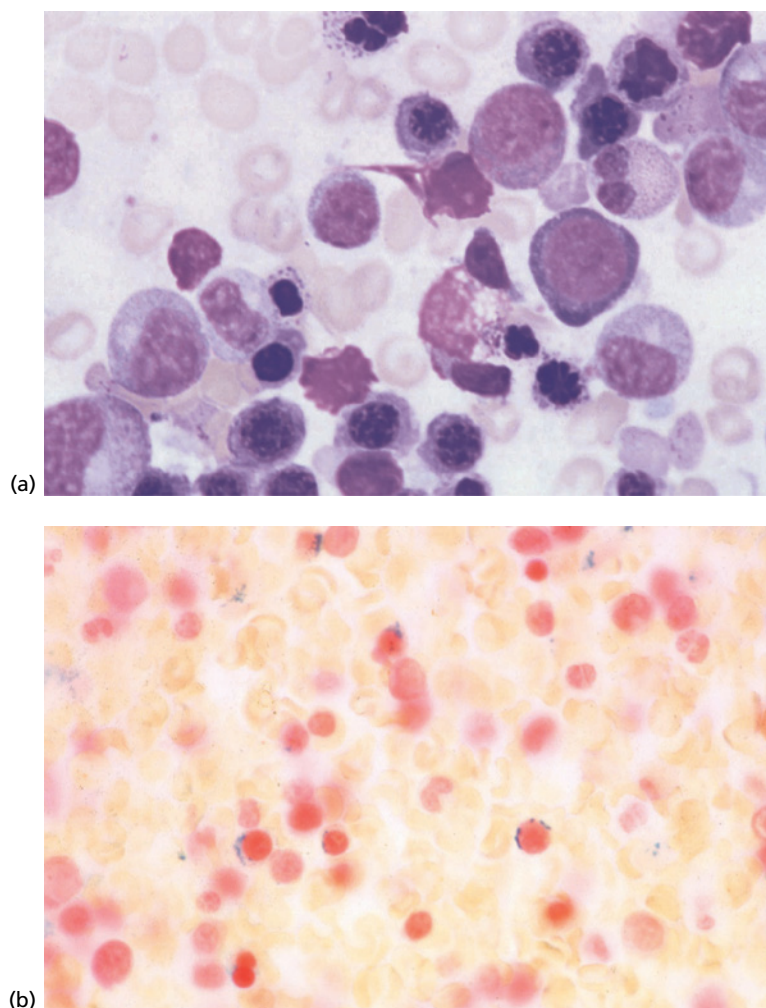


Fig. 5.51 Bone marrow aspirate in haemoglobin St Mary's: (a) MGG $\times 100$ showing erythroid hyperplasia and dyserythropoiesis (lobulated nuclei and basophilic stippling); (b) Perls stain $\times 100$ showing ring sideroblasts.

chain of glutamic acid, substituted for valine at $\beta 68$, reaches the haem molecule and the ferric state is stabilised [127]. Once the variant globin chain has been oxidised it becomes non-functional from the point of view of oxygen transport. In the case of the three α chain variants, M-Boston, M-Iwate and M-Milwaukee, only the β chains can bind oxygen; in the case of the two β chain variants, M-Saskatoon and M-Hyde Park, only the α chains can bind oxygen. Conversion to methaemoglobin may be accelerated by exogenous oxidants. Some M haemoglobins have a reduced oxygen affinity.

Some are also unstable and instability may be aggravated by oxidant stress. Haemoglobin M-Hyde Park is an example of an unstable haemoglobin M with partial haem loss leading to haemoglobin instability and haemolysis. Haemoglobin M-Saskatoon is also associated with haemolysis and jaundice. Haemoglobin Chile is an unstable haemoglobin associated with chronic methaemoglobinemia [145]. Methaemoglobins all have a marked reduction in cooperativity indicated by a low n number [147] (see later). These variant haemoglobins are summarised in Table 5.3 [2, 147–153].

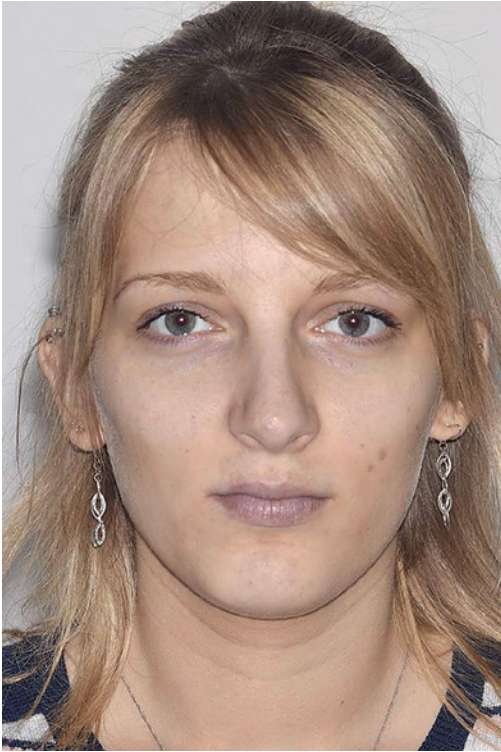


Fig. 5.52 Clinical photograph of a patient with haemoglobin M-Saskatoon showing pseudocyanosis. (With thanks to Dr Thomas Erblich and the patient.)

The α chain M haemoglobins investigated to date have all been $\alpha 1$ variants [2]. It has been postulated that this is because, with an $\alpha 2$ mutation, the preferential combination of the variant α chain with normal non- α chains could lead to a haemoglobin M percentage incompatible with fetal viability.

The differential diagnosis includes inherited methaemoglobinaemia due to an enzyme deficiency and acquired methaemoglobinaemia due to oxidant stress.

Clinical features

There is cyanosis from birth in the case of α chain variants and from around 3–6 months of age in the case of β chain variants. Babies with γ chain variants (haemoglobin F-M-Osaka, haemoglobin F-M-Fort Ripley and haemoglobin F-Circleville) are mildly cyanosed at birth but cyanosis lessens as β chain production takes over from γ [119]; such babies may require supplemental oxygen in the first few months of life. When there is a significant amount of methaemoglobin present, a blood sample appears macroscopically brownish. Treatment with methylene blue is sometimes used to reduce methaemoglobin levels in enzyme deficiencies

Fig. 5.53 Diagram showing the relationship of haem to the proximal and distal histidines of the haem pocket; mutation of either of the corresponding codons can lead to a haemoglobin M.

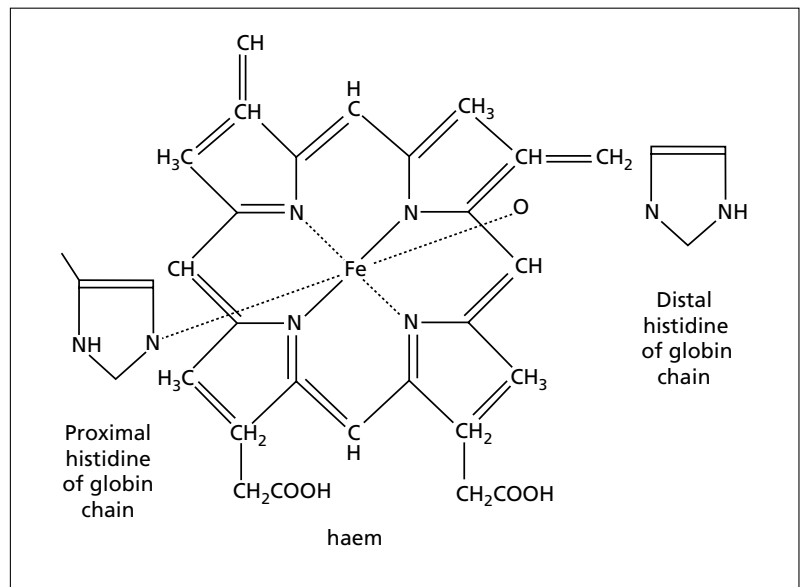


Table 5.3 M haemoglobins [2, 147–153].

Haemoglobin	Oxygen affinity	Usual percentage of variant	<i>n</i> number*	Other features
β chain variants				
Haemoglobin M-Saskatoon (α ₂ β ₂ ^{63 [E7] His→Tyr})	Normal	35%	1.2	Mild or no haemolysis
Haemoglobin M-Hyde Park (α ₂ β ₂ ^{92 [F8] His→Tyr})†	Normal	NA	1.3	Mild haemolysis
Haemoglobin M-Milwaukee (α ₂ β ₂ ^{67 [E11] Val→Glu})	Reduced	50%	1.2	No haemolysis
Haemoglobin Chile (α ₂ β ₂ ^{28 Leu→Met})				Cyanosis
α chain variants				
Haemoglobin M-Iwate (α ₂ ^{87 [F8] His→Tyr} β ₂)‡	Reduced	19%	1.1	Not anaemic
Haemoglobin M-Boston (α ₂ ^{58 [E7] His→Tyr} β ₂ or α ₁ ^{175C→T} β ₂)	Reduced	NA, 7.6%	1.2	Sometimes anaemic
γ chain variants				
Haemoglobin F M-Osaka (α ₂ γ ₂ ^{63 [E7] His→Tyr})		5–8%, 12%, 17.5%		Neonatal cyanosis [148]
Haemoglobin F M-Fort Ripley (α ₂ γ ₂ ^{92 [F8] His→Tyr})	Slightly increased	10%, 9%		Neonatal cyanosis, also unstable [149]
Haemoglobin F-Circleville (α ₂ γ ₂ ^{63 [E7] His→Leu})§	Normal	14%		Neonatal cyanosis [150]
Haemoglobin F M-Viscu (Hb Shady Grove) (α ₂ γ ₂ ^{28 Leu→Met})		16%, 7–15%		Neonatal cyanosis [151, 152]

NA, not available.

* The *n* number indicates the degree of cooperativity between haemoglobin subunits; normal haemoglobins have an *n* value of 2.7–3.0 whereas a haemoglobin with no cooperativity has an *n* value of 1 [147].

† Sometimes called haemoglobin Milwaukee II.

‡ Also known as Hb Kankakee, Hb M-Oldenberg and Hb M-Sendai [2].

§ Presumptive identification as a haemoglobin M.

and acquired methaemoglobinaemia, although this has little or no effect on the methaemoglobin levels associated with a haemoglobin M.

Laboratory features

Blood count

The Hb may be normal or high. Occasional patients are anaemic. The reticulocyte count is sometimes elevated, particularly in the case of carriers of haemoglobin M-Hyde Park and haemoglobin M-Saskatoon, which may be associated with a compensated haemolytic state.

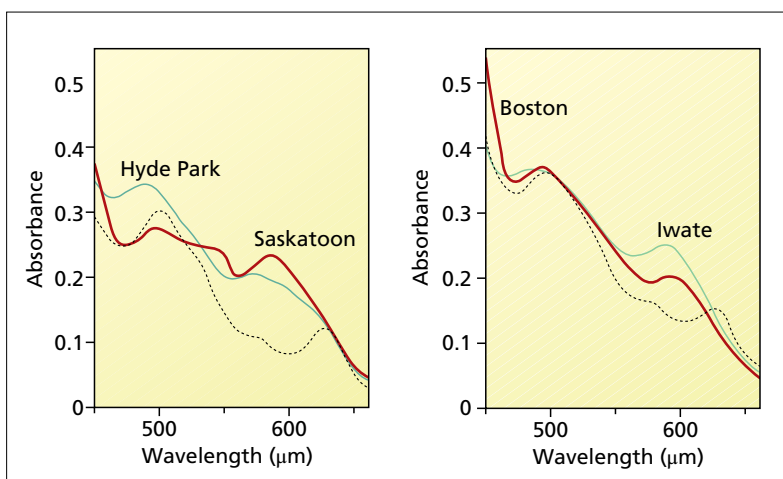
Blood film

The blood film can show poikilocytosis.

Other investigations

Diagnosis is usually by spectrometry since the absorbance spectrum of methaemoglobin differs from that of haemoglobin A. Methaemoglobin is also commonly detected by most blood gas analysers, and the diagnosis may first be made in this way. The absorption spectra of different haemoglobin Ms differ from each other but not sufficiently to permit definitive diagnosis. However,

Fig. 5.54 Absorption spectra of haemoglobin M-Iwate, M-Boston, M-Hyde Park and M-Saskatoon in comparison with methaemoglobin A. ([1]/with permission of Elsevier.)



if haemoglobin is first oxidised the spectra are characteristic (Fig. 5.54). Haemoglobin electrophoresis is normal at alkaline pH but electrophoresis at a pH of 7 in a phosphate buffer shows abnormal mobility of haemoglobin Ms. since the mutation involved histidine, which ionises at neutral pH [154]. Other congenital and acquired causes of methaemoglobinaemia need to be excluded (see page 389). In contrast to low affinity haemoglobins, oxygenation of the sample *in vitro* does not restore the normal colour. Haemoglobin electrophoresis may show an abnormal band but the normal and variant haemoglobins are more readily separated if they are both converted to methaemoglobin prior to electrophoresis. The oxygen dissociation curve can be abnormal in shape and either right or left shifted. A test for haemoglobin instability should be performed since some unstable haemoglobins also show accelerated formation of methaemoglobin. Heinz bodies may be present, particularly in the case of haemoglobin M-Saskatoon. Pulse oximetry is inaccurate in the presence of methaemoglobin, because the absorption pattern of methaemoglobin interferes with the algorithm used to calculate oxygen saturation. When significant amounts of methaemoglobin are present, most pulse oximeters give a fixed reading of about 85%, which does not reflect the underlying oxygen saturation, which may actually be higher or lower; co-oximetry may give more meaningful results [155, 156].

High affinity haemoglobins

The presence of a variant haemoglobin with a high oxygen affinity usually leads to polycythaemia. The exception is when a high affinity haemoglobin is also very unstable, as is the case with haemoglobin Köln. About a third of unstable haemoglobins show an increased oxygen affinity but since the dominant feature is haemolysis they are categorised primarily as unstable haemoglobins rather than as high affinity haemoglobins. Some high affinity haemoglobins also produce a thalassaemic phenotype (e.g. haemoglobin Crete, which is associated with marked microcytosis and unbalanced chain synthesis) [157]. The first high affinity haemoglobin recognised was an α chain variant, haemoglobin Chesapeake, described by Charache, Weatherall and Clegg in 1966 [158].

The diagnosis of a high affinity haemoglobin should be considered when there is unexplained polycythaemia, particularly in a young person or when a patient with a high Hb has a family history of polycythaemia. It is important that patients with polycythaemia resulting from a high affinity haemoglobin are not misdiagnosed as polycythaemia vera. In the past, such misdiagnosis led to exposure to ^{32}P , for up to 10 years, and even to ^{32}P -induced myelodysplastic syndrome or acute myeloid leukaemia [1, 159]. Onset of a haematological abnormality is from birth in the case of an α chain variant and during the first year of life in the case of a β chain variant.

More than 120 high affinity variant haemoglobins have been described. In most cases there is only a single copy of the mutant gene but in the case of the α variants, haemoglobin Tarrant and haemoglobin Longview, individuals with two copies of the gene have been described and compound heterozygosity for haemoglobin Tarrant and haemoglobin Jackson has also been recognised [160]. Homozygosity for the β chain variant, haemoglobin Abruzzo, has likewise been described [160]. Compound heterozygosity for a β chain variant and β^0 thalassaemia can occur, and leads to a more severe phenotype. The molecular abnormality may result in:

- interference with the $\alpha_1\beta_2$ contacts, which normally allow movement of the haemoglobin subunits in relation to each other on oxygenation, with stabilisation of the R (oxy) conformation or destabilisation of the T (deoxy) conformation;
- interference with $\alpha_1\beta_1$ contacts with major disruption of the quaternary structure and favouring of the R (oxy) conformation;
- limited polymerisation, restraining the quaternary conformation and favouring the R (oxy) conformation;
- reduced binding to 2,3-DPG;
- abnormality of the amino end of the α chain or of either the amino or carboxy end of the β chain, leading to disruption of the quaternary structure and favouring of the R (oxy) conformation;
- alteration of the haem pocket.

Occasional high affinity haemoglobins (e.g. Hb Headington) have a mutation leading to alteration of a surface amino acid residue; the molecular mechanism of the altered affinity is not clear [161]. Haemoglobin Olympia, which also has a substitution affecting a surface amino acid, has now been shown to be characterised by self-aggregation of haemoglobin tetramers [127].

High affinity haemoglobins often have reduced cooperativity and a reduced Bohr effect and some are unstable.

The molecular abnormalities responsible include not only single point mutations but also double point mutations (haemoglobin Poissy, only one of the mutations is relevant), deletions, insertions and frameshift mutations (haemoglobin Tak) [127].

A number of high affinity haemoglobins form stable hybrid tetramers (with both a normal and a variant β chain) leading to a broad peak on HPLC and two variant bands on electrophoresis [162].

Clinical features

The individual with a high affinity haemoglobin may be plethoric but otherwise well. In later life it is likely that the risk of thrombosis is increased. In contrast to polycythaemia vera, there is no hepatomegaly or splenomegaly. Symptoms of hyperviscosity – headache, vertigo, tinnitus and paraesthesia – can occur [163]. Serum erythropoietin is normal when the patient is in a stable state but is increased by venesection. In one study, neither the Hb/haematocrit nor treatment by venesection showed a correlation with thrombosis or with symptomatic disease, suggesting a limited role for venesection [164]. Theoretical considerations would suggest that transfer of oxygen to the fetus might be impaired when a woman has a high affinity haemoglobin but in practice pregnancy is generally uneventful.

Laboratory features

Blood count

The RBC, Hb and haematocrit are high in the normal range or elevated but other red cell indices are normal. Whether the Hb is above the normal range is determined by:

- the degree of shift of the oxygen dissociation curve (i.e. the $P_{50}O_2$);
- the percentage of the variant haemoglobin (determined in turn by whether the variant haemoglobin is an α or a β variant and whether it is also unstable and whether there is coexisting thalassaemia).

Somewhat surprisingly, high affinity haemoglobins have been associated not only with a true polycythaemia but also with a relative polycythaemia (i.e. with a reduced plasma volume but normal red cell mass) [165]. The white cell count and the platelet count are usually normal but some individuals have been reported with an elevated white cell count [166].

Blood film

The blood film appears 'packed' because the high haematocrit and increased whole blood viscosity lead to a thick film.

Other laboratory features

The total red cell mass, as measured by radioisotope dilution techniques, is raised in relation to what is normal for an individual of the same gender, height and weight. The plasma volume can be normal or reduced. The partial pressure of oxygen in arterial blood is normal.

Haemoglobin electrophoresis on cellulose acetate at alkaline pH sometimes shows a haemoglobin variant with abnormal electrophoretic mobility but this is not necessarily so. Electrophoresis at acid pH can be useful when electrophoresis at alkaline pH is normal as sometimes a variant haemoglobin is revealed only at acid pH. About three-quarters of unstable haemoglobins can be detected by either electrophoresis or isoelectric focusing. HPLC or mass spectrometry permits detection of other high affinity haemoglobins [167]. Determining the $P_{50}O_2$ on a blood gas analyser can be used for screening for a high affinity haemoglobin and should be done whenever there is a suspicion of a high affinity haemoglobin, even if no electrophoretically abnormal variant or HPLC abnormality has been detected. If an abnormality is detected, an oxygen dissociation curve can be performed. If a relevant abnormality is found and no variant haemoglobin has been detected by other techniques, sequencing of the globin genes is indicated. The variant haemoglobin is usually around 50% in the case of a β chain variant and 12–40% in the case of an α chain variant. The percentage of β chain variants is higher if there is coexisting β thalassaemia in *trans*, being almost 100% in the case of coexisting β^0 thalassaemia. The percentage of α chain variants is similarly greater if there is coexisting α thalassaemia.

The oxygen dissociation curve is left shifted and the $P_{50}O_2$ is decreased (Fig. 5.55). Reported $P_{50}O_2$ has ranged from as low as 9.5 mmHg (haemoglobin Heathrow), 10 mmHg (haemoglobin McKees Rocks) or 11 mmHg (haemoglobin

Syracuse) to slightly below normal. Since heterozygosity is usual, the oxygen dissociation curve is often biphasic, representing the mixture of normal and variant haemoglobins. In other cases the entire dissociation curve is shifted, possibly because of the presence of hybrid haemoglobin molecules (i.e. $\alpha_2\beta^A\beta^X$) [168]. The normal sigmoid shape of the oxygen dissociation curve is usually lost to a variable extent, this being quantified by the n value. This is calculated according to Hill's equation: $KP^n = [Y/(1-Y)]$ where K is a constant, P is the partial pressure of oxygen and Y is the fractional oxygen saturation. The log of $Y/(1-Y)$ plotted against the log of PO_2 normally gives a straight line but, when the oxygen dissociation curve is biphasic, the line is bent. The value of n when cooperativity is normal is about 2.6–2.7 whereas n is approximately 1 when there is no cooperativity. High affinity haemoglobins typically have n values of 1.1–1.9.

Concentration of 2,3-DPG is increased. The serum erythropoietin concentration is normal or high but if the individual is venesected to

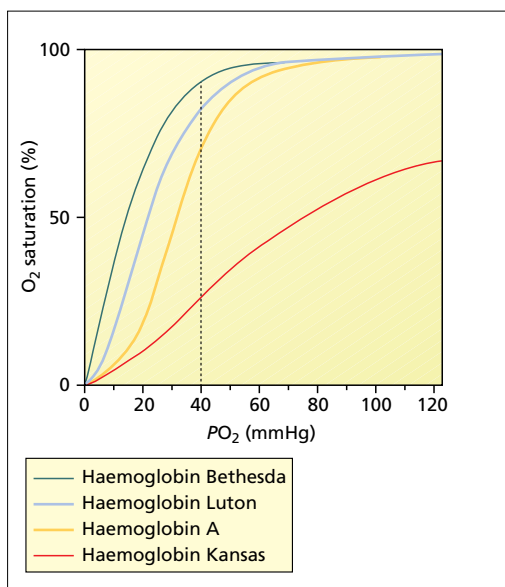


Fig. 5.55 Oxygen dissociation curve showing two high affinity haemoglobins and one low affinity haemoglobin in comparison with haemoglobin A; the vertical dotted line shows the normal partial pressure of oxygen in venous blood.

lower the Hb, a marked rise of erythropoietin concentration occurs.

Diagnosis

At least two standard electrophoretic or HPLC techniques are recommended, in addition to an oxygen dissociation curve and measurement of $P_{50}O_2$. A test for an unstable haemoglobin should also be carried out in patients with unexplained polycythaemia since some high affinity haemoglobins are unstable. Sequencing of the globin genes is increasingly used as an early test in the investigation of polycythaemia and possible high affinity haemoglobins, and may be used to direct further phenotypic studies, such as measurement of haemoglobin oxygen affinity.

Coinheritance of a high affinity haemoglobin and other variant haemoglobins or thalassaemia

Coinheritance of a high affinity β chain variant haemoglobin and $\delta\beta^0$ thalassaemia has been observed to aggravate the polycythaemia, a result that would be predicted since the only haemoglobins present are the variant (Hb Headington or haemoglobin Tak) and haemoglobin F, which also has a high oxygen affinity [161, 169]. Coinheritance of a high affinity haemoglobin and β^0 thalassaemia can similarly result in more marked polycythaemia than in the simple heterozygous state for the variant haemoglobin [170]. Coinheritance of β thalassaemia and the high affinity β chain variants, haemoglobin Crete and haemoglobin San Diego did not prevent the development of polycythaemia [23]. Coinheritance of haemoglobin Tak with haemoglobin E was associated with a mild polycythaemia [89].

Low affinity haemoglobins

At least 70 low affinity haemoglobins have been described. Some variant haemoglobins that primarily cause other syndromes also have a low oxygen affinity, so that a low Hb in homozygous states is partly consequent on better oxygen delivery to tissues with a lessening

of erythropoietic drive. This is true of sickle cell disease. Some unstable haemoglobins also have reduced oxygen affinity so that a low Hb is well tolerated. If the instability is mild, as in the case of haemoglobin Kansas, the only clinical features may be a well tolerated anaemia; when affinity is markedly reduced, there can be cyanosis. An unexplained association of low affinity haemoglobins with pulmonary hypertension has been reported in three families [171–173]. More low affinity β chain than α chain variants have been described, probably because the lower percentage of an α chain variant means that its effects are less likely to be noticed. In the neonatal period, low affinity γ chain variants, such as haemoglobin F-Heuried, which is also unstable [174], and haemoglobin F-Cincinnati [175], can lead to cyanosis. Incidental diagnosis, particularly in neonates, can result from detection of a low O_2 saturation despite normal arterial blood gases [176]. The rate of detection of low affinity haemoglobins is likely to have increased in the USA with the introduction of screening for cyanotic congenital heart disease by pulse oximetry [177].

Variant haemoglobins with a moderate decrease in oxygen affinity are characterised by anaemia whereas those with a marked decrease in affinity (whole blood $P_{50}O_2$ greater than 50 mmHg) are characterised by cyanosis. Cyanosis has been observed, for example, with haemoglobins Kansas, Beth Israel, St Mande [1], Titusville [178] and Bassett [179]. In the case of an α chain variant, the cyanosis is present from birth whereas with a β chain variant it appears during the first year of life. O_2 saturation is reduced. If the variant haemoglobin is a low percentage, the Hb may fall within the normal range [178] and cyanosis may be absent. Tissue extraction of O_2 does not have a linear relationship to $P_{50}O_2$; initially it increases as P_{50} increases but then decreases so that by a whole blood P_{50} of 80 mmHg, oxygen extraction is again normal [178].

The mechanism of reduced oxygen affinity [127, 180] may be:

- alteration of the $\alpha_1\beta_2$ interface that either stabilises the T (deoxy) conformation (e.g. haemoglobin Titusville) or destabilises the R (oxy) conformation (e.g. haemoglobin Kansas);

- alteration of the $\alpha_1\beta_1$ contacts that disrupt the molecule, favouring the T (deoxy) conformation (e.g. haemoglobin Presbyterian);
- increased affinity for 2,3-DPG (e.g. haemoglobin Aalborg);
- steric hindrance in the haem environment (e.g. haemoglobin Bologna);
- alteration of the N-terminal residue of the α chain with resultant stabilisation of the deoxy form (e.g. haemoglobin Thionville) or abnormal interaction with chloride ions, favouring the deoxy form (haemoglobin Lyon-Bron) [180].

An increased concentration of 2,3-DPG can contribute to the low affinity, since desaturation leads to a rise in pH which increases the synthe-

sis of 2,3-DPG [127]. Low affinity haemoglobins sometimes have reduced cooperativity or a reduced Bohr effect.

Other α and β chain variants

Other variant haemoglobins that may be encountered include the α chain variant, haemoglobin Q-Iran (Fig. 5.56) and the β chain variant, haemoglobin D-Iran (Fig. 5.57), which need to be distinguished from variant haemoglobins of clinical significance. Haemoglobin G-Makassar, a rare β chain variant found in Thailand and Malaysia, resembles haemoglobin S on alkaline and acid electrophoresis and

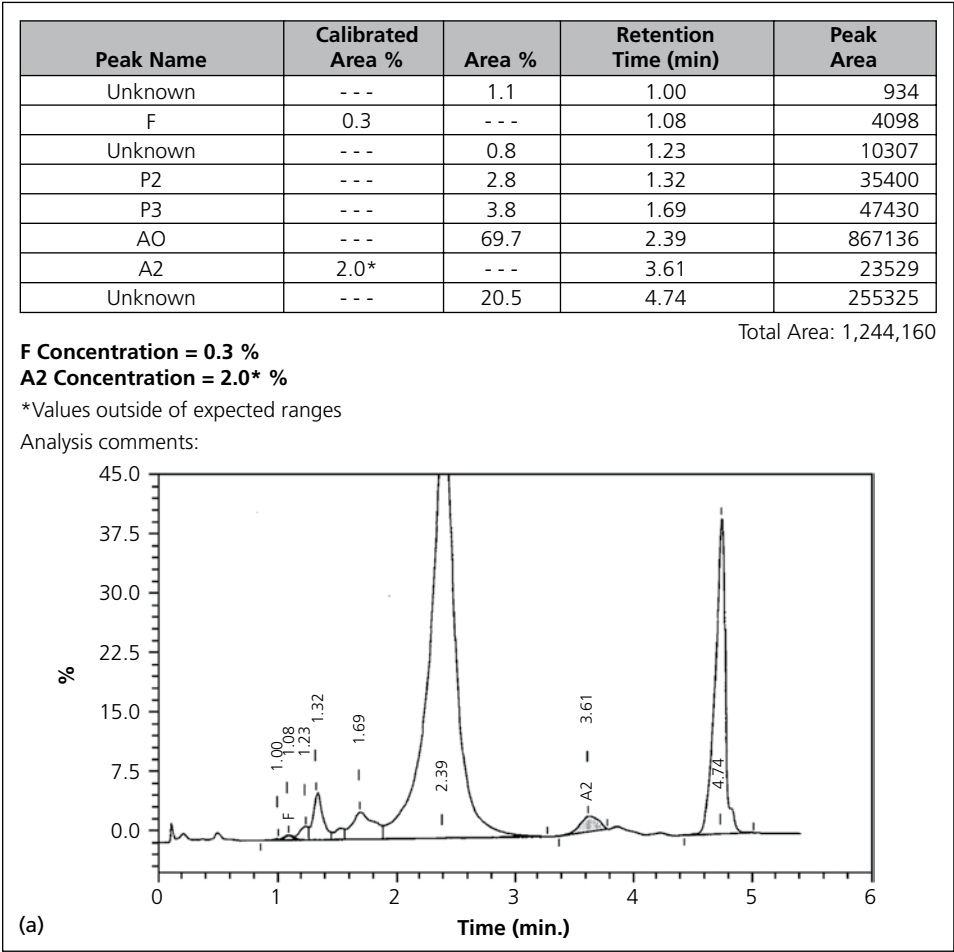


Fig. 5.56 Haemoglobin Q-Iran: (a) HPLC (Bio-Rad variant II) showing two major peaks representing haemoglobins A₀ and Q-Iran; (Continued on p. 362.)

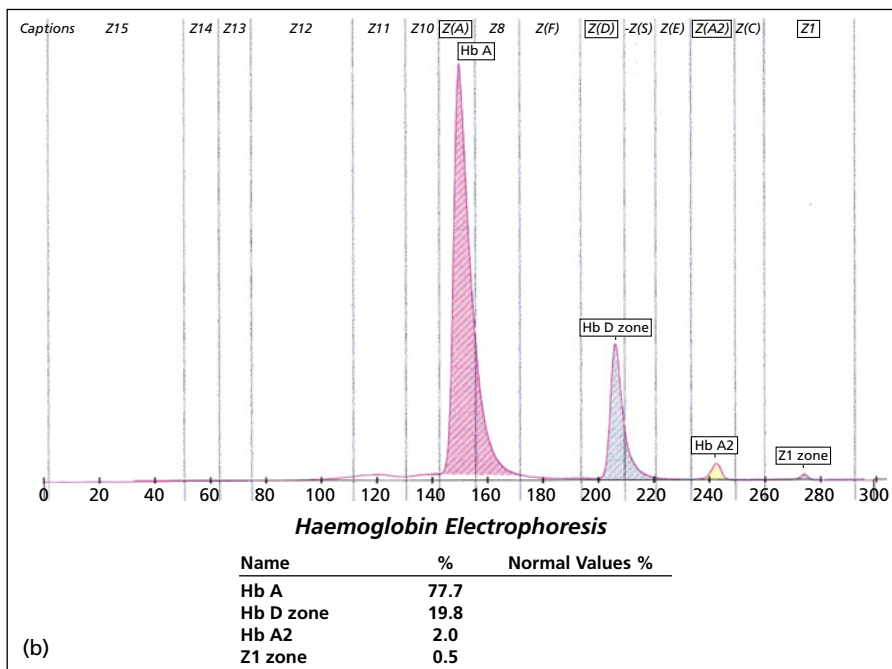


Fig. 5.56 Continued. (b) capillary electrophoresis (Sebai Capillaries) showing the variant haemoglobin in the D zone.

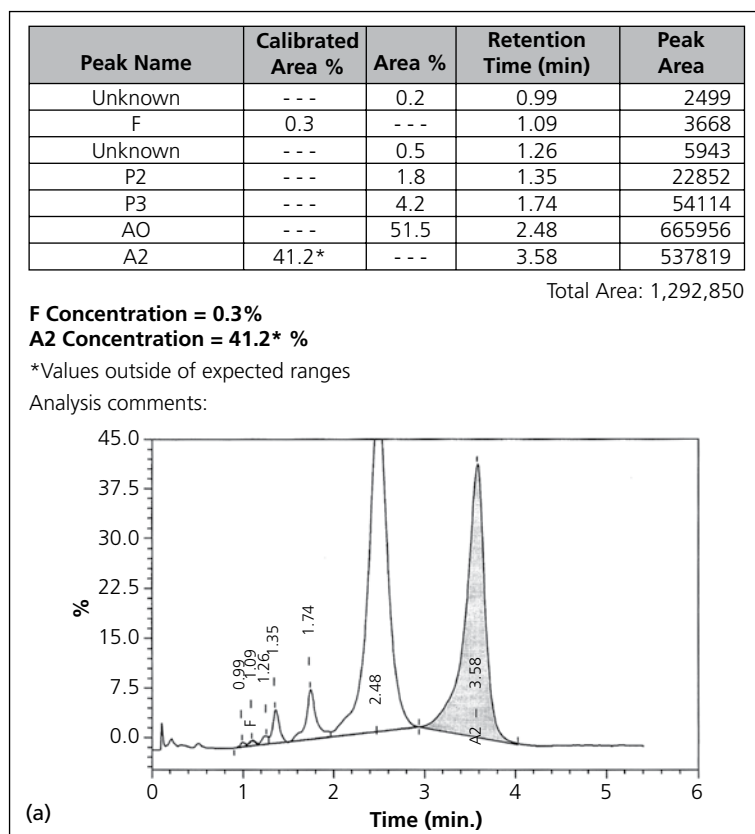


Fig. 5.57 Haemoglobin D-Iran: (a) HPLC (Bio-Rad variant II) showing two major peaks representing haemoglobins A₀ and D-Iran (in the A₂ window);

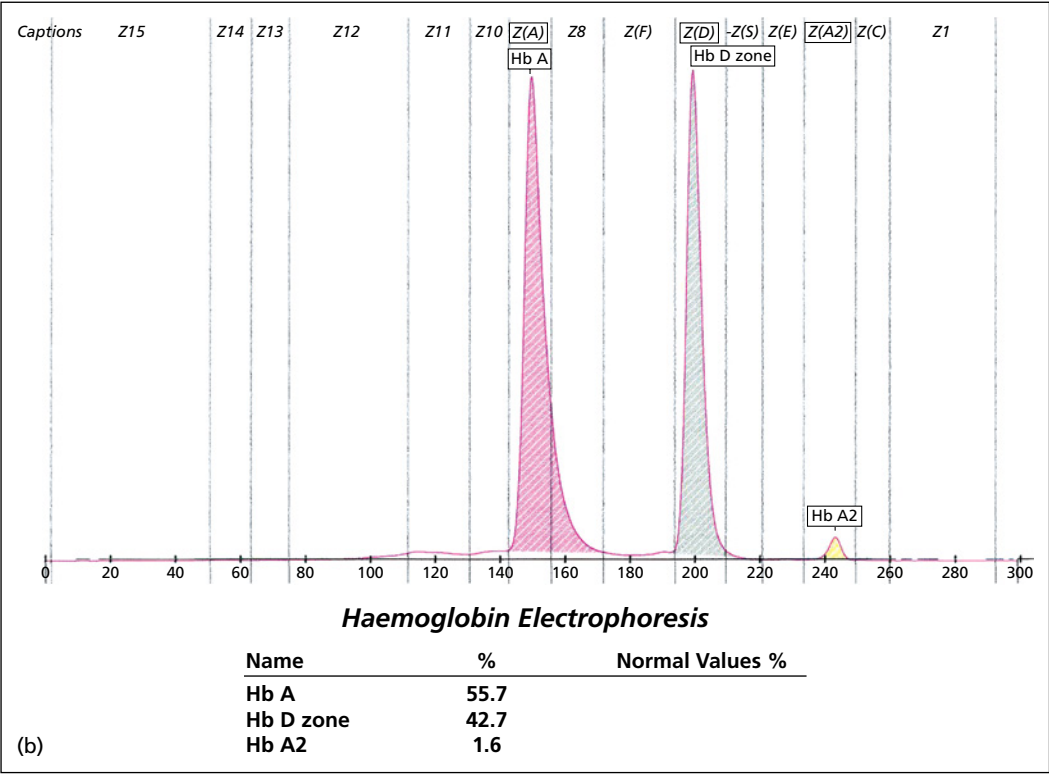


Fig. 5.57 Continued. (b) capillary electrophoresis (Sebia Capillars) showing the variant haemoglobin in the D zone.

HPLC; red cell indices resemble those of thalassaemia trait [181]. Base editing to correct the sickle mutation in gene therapy results in haemoglobin G-Makassar.

Haemoglobin F variants

At least 69 haemoglobin F variants are known, somewhat more being γ variants than α variants [119]. In the neonatal period most γ variants constitute around 31% of total haemoglobin while α variants are around 13% [119]. Several haemoglobin F variants have two amino acid substitutions. Many of these are not of any clinical significance but there are several unstable fetal methaemoglobins, which can cause cyanosis in the neonatal period. One $\gamma\alpha$ fusion gene leads to γ thalassaemia. Several haemoglobin F variants are M haemoglobins (see earlier).

Haemoglobin A₂ variants

More than 20 haemoglobin A₂ variants (Fig. 5.58) are known [182]. They are not of any clinical significance but can complicate the diagnosis of β thalassaemia trait since their presence can lead to a failure to measure the total of haemoglobin A₂ plus haemoglobin A₂ variant (Figs 5.59 and 5.60). The most common of these variants is haemoglobin A₂' (initially known as haemoglobin B₂). It originated in Africa and occurs in between 1% and 2% of African Americans (1.6% overall of 14321 individuals in nine studies [183]). Its highest known frequency is among the Herero people of Namibia where heterozygotes are more than 18% of the population [184]. It also occurs with polymorphic frequency in the Dogon region of Mali, being found overall in 2.5% of the population but in up to 12% in one caste group [185]. Other A₂ variants are common in very specific ethnic groups; for example, approaching 5% of

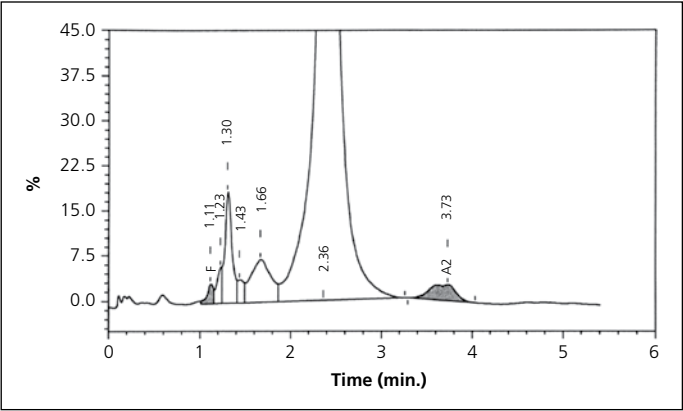


Fig. 5.58 HPLC chromatogram (Bio-Rad Variant II) showing a double peak resulting from the presence of a haemoglobin A₂ variant; both the variant and the normal A₂ appeared within the A₂ window so that quantification was accurate, whereas the most frequently observed A₂ variant, A₂' (see Fig. 5.59), is quantified as haemoglobin S; peaks, from left to right, are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobin A₀ and haemoglobins A₂ plus A₂ variant (double peak, shaded).

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.7	---	1.05	7954
Unknown	---	0.7	1.23	9006
P2	---	4.0	1.33	51513
P3	---	4.2	1.70	53756
AO	---	85.4	2.36	1103702
A2	3.5*	---	3.67	46235
S window	---	1.6	4.58	20432

Total Area: 1,292,598

F Concentration = 0.7 %

A2 Concentration = 3.5* %

*Values outside of expected ranges

Analysis comments:

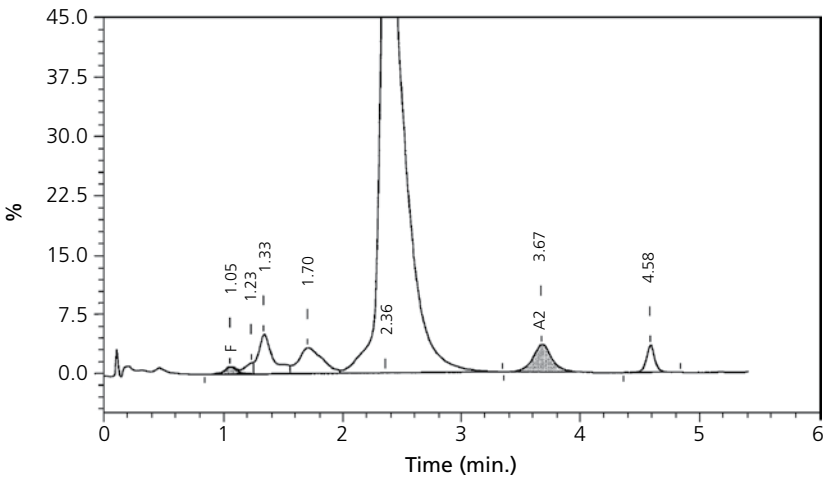


Fig. 5.59 HPLC chromatogram (Bio-Rad Variant II) in a patient with both haemoglobin A₂' and β thalassaemia heterozygosity. The peaks from left to right are haemoglobin F (shaded), post-translationally modified haemoglobin A (two peaks), haemoglobin A₂ (shaded) and haemoglobin A₂' (in S window). Note that the normal A₂ peak is 3.5% but if the A₂' prime peak is added the total is 5.1% and the diagnosis of β thalassaemia heterozygosity becomes evident.

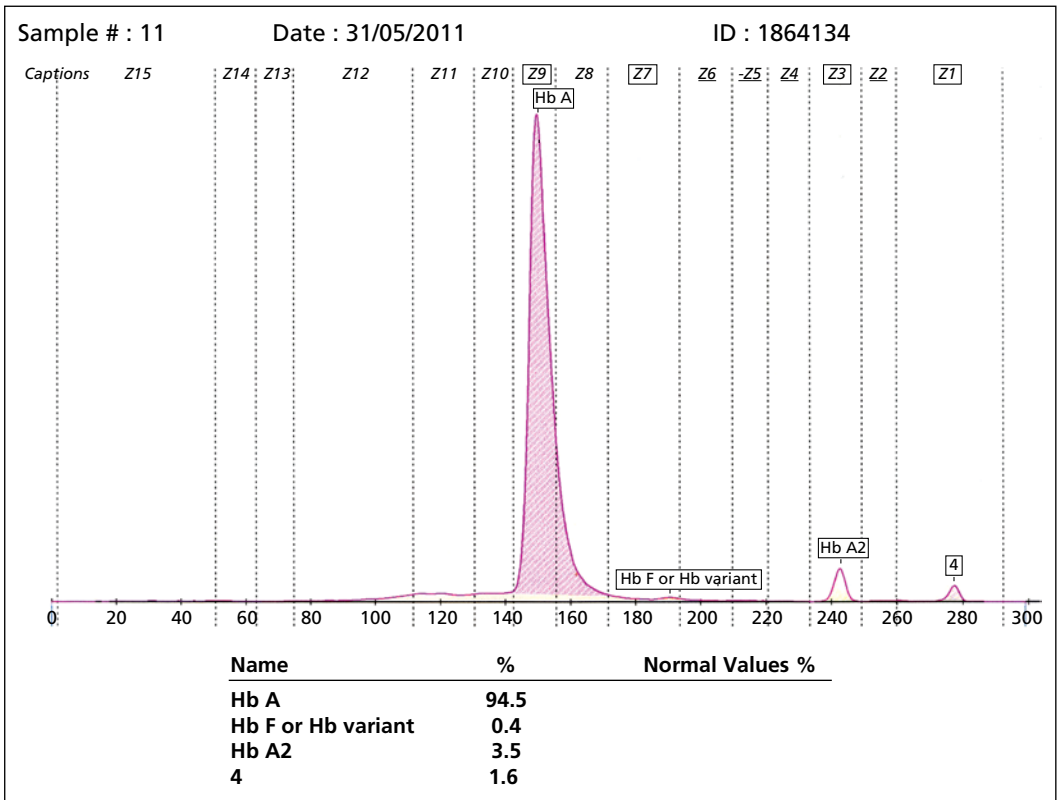


Fig. 5.60 Capillary electrophoresis (Sebia Capillars 3) in a patient with both haemoglobin A₂' and β thalassaemia heterozygosity. Note that the normal A₂ peak is 3.5% but if the A₂' prime peak (labelled 4) is added, the total is 5.1%. Same patient as Fig. 5.59.

Babinga pygmies in the Central African Republic carry haemoglobin A₂-Flatbush and a similar percentage of Sumatrans carry haemoglobin A₂-Indonesia [171]. Most A₂ variants are synthesised at an approximately normal rate. Several very rare variants are unstable and therefore present at a very low percentage [171].

Check your knowledge

One to five answers may be correct. Answers to almost all questions can either be found in this chapter or can be deduced from information given. Answers are given on page 376.

- 5.1 Haemoglobin C homozygosity is usually associated with
- an increased reticulocyte count
 - an increased incidence of gallstones

- a need for an increased iron intake to prevent anaemia
- a blood film showing target cells and irregularly contracted cells
- approximately equal proportions of haemoglobins A and C

5.2 Haemoglobin E

- has its highest prevalence in South-East Asia
- can interact adversely with β thalassaemia
- is synthesised at a reduced rate because of a splicing defect leads to impaired production of mRNA
- is unstable *in vitro*
- can be confused with haemoglobin C on agarose gel electrophoresis at pH6.5

- 5.3 Haemoglobin G-Philadelphia
 - (a) is of major clinical significance because of its interaction with haemoglobin S
 - (b) has arisen by at least two independent mutations
 - (c) is associated with a variant form of haemoglobin A₂
 - (d) has a high oxygen affinity
 - (e) moves with haemoglobin S on electrophoresis at alkaline pH and with haemoglobin A on electrophoresis at acid pH
- 5.4 An unstable haemoglobin
 - (a) may have increased oxygen affinity
 - (b) may have decreased oxygen affinity
 - (c) may be electrophoretically silent
 - (d) may be prone to conversion to methaemoglobin
 - (e) usually only causes symptoms in homozygotes
- 5.5 Haemoglobin O-Arab
 - (a) is an α chain variant
 - (b) is the most common variant haemoglobin in Arabs
 - (c) can interact with haemoglobin S to cause a clinically severe disease
 - (d) is electrophoretically silent
 - (e) has a significantly increased oxygen affinity
- 5.6 A high oxygen affinity haemoglobin
 - (a) usually causes anaemia
 - (b) is associated with a reduced serum erythropoietin
 - (c) usually compromises the outcome of pregnancy because of reduced oxygen delivery to the fetus
 - (d) is associated with a left-shifted oxygen dissociation curve
 - (e) may also be unstable
- 5.7 Heterozygotes for haemoglobin E
 - (a) often have 25–30% of the variant haemoglobin
 - (b) occasionally have a normal blood film and red cell indices
 - (c) usually have West African ancestry
 - (d) can have red cell indices similar to those of thalassaemia trait
 - (e) on cellulose acetate electrophoresis at alkaline pH can be readily confused with β thalassaemia trait
- 5.8 Irregularly contracted cells can be a feature of
 - (a) β thalassaemia trait
 - (b) homozygosity for haemoglobin C
 - (c) homozygosity for haemoglobin E
 - (d) acute chest syndrome in sickle cell anaemia
 - (e) an unstable haemoglobin
- 5.9 Haemoglobin D-Punjab (D-Los Angeles)
 - (a) occurs in a small proportion of Africans and African Caribbeans
 - (b) is of no clinical or genetic significance
 - (c) is an α chain variant and is therefore associated with a split haemoglobin A₂ band on cellulose acetate electrophoresis
 - (d) has its highest prevalence in southern India
 - (e) on cellulose acetate electrophoresis at alkaline pH can easily be confused with haemoglobin S
- 5.10 Reduced red cell survival is characteristic of
 - (a) Homozygosity for haemoglobin C
 - (b) Heterozygosity for haemoglobin Köln
 - (c) Homozygosity for haemoglobin E
 - (d) Heterozygosity for haemoglobin O-Arab
 - (e) Haemoglobin H disease
- 5.11 An unstable haemoglobin may be associated with
 - (a) Heinz body haemolytic anaemia
 - (b) a positive isopropanol test
 - (c) oxidant-induced haemolysis
 - (d) an abnormal band on haemoglobin electrophoresis representing haem-depleted haemoglobin
 - (e) splenomegaly

- 5.12 Cyanosis may be caused by heterozygosity for
 - (a) haemoglobin C
 - (b) haemoglobin D
 - (c) haemoglobin E
 - (d) haemoglobin M
 - (e) low oxygen affinity haemoglobins
- 5.13 The percentage of a variant haemoglobin is determined by
 - (a) whether it is an α or β chain variant
 - (b) the oxygen affinity of the variant haemoglobin
 - (c) whether there is coexisting thalassaemia
 - (d) the affinity of the variant chain for normal α or β chain
 - (e) whether the variant chain is synthesised at a normal or reduced rate

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Answers to questions

- | | | | | |
|---|---|---|--|--|
| 5.1 (a) T
(b) T
(c) F
(d) T
(e) F | 5.4 (a) T
(b) T
(c) T
(d) T
(e) F | 5.7 (a) T
(b) T
(c) F
(d) T
(e) F | 5.10 (a) T
(b) T
(c) F
(d) F
(e) T | 5.13 (a) T
(b) F
(c) T
(d) T
(e) T |
| 5.2 (a) T
(b) T
(c) T
(d) T
(e) F | 5.5 (a) F
(b) F
(c) T
(d) F
(e) F | 5.8 (a) T
(b) T
(c) T
(d) T
(e) T | 5.11 (a) T
(b) T
(c) T
(d) T
(e) T | |
| 5.3 (a) F
(b) T
(c) T
(d) F
(e) T | 5.6 (a) F
(b) F
(c) F
(d) T
(e) T | 5.9 (a) T
(b) F
(c) F
(d) F
(e) T | 5.12 (a) F
(b) F
(c) F
(d) T
(e) T | |

6 Acquired abnormalities of globin chain synthesis or haemoglobin structure

Acquired disorders of globin chain synthesis can result from (i) clonal erythropoiesis including variants or deletions of globin genes; (ii) altered methylation status of a globin gene leading to altered expression; (iii) the influence of other genes on the expression of globin genes; or (iv) abnormalities influencing the bone marrow niche, including metabolic changes and stress erythropoiesis. Acquired somatic mutations of globin genes are very rare. Altered expression is much more common.

Post-translational modification of haemoglobin structure can also occur as a result of inherited abnormalities, other than those of globin genes, or as a result of exposure to toxic drugs or chemicals.

Acquired thalassaemia

Alterations in the rates of globin chain synthesis, with $\alpha:\beta$ ratios similar to those observed in thalassaemia, are quite common in myeloid malignancies. This could be regarded as a mild form of acquired thalassaemia. Peters et al. [1] observed an increased $\alpha:\beta$ ratio in six of 11 patients with myelodysplastic syndromes (MDS) ($\alpha:\beta$ chain synthesis ratios of 1.28–2.43, normal range 0.97–1.19) and in two of four patients with acute myeloid leukaemia (AML) (both evolved from MDS, $\alpha:\beta$ chain synthesis ratios of 1.8 and 2.1). In this study, red cell hypochromia and microcytosis were not confined to patients with abnormalities in the $\alpha:\beta$ chain synthesis ratio.

The phenotype of α or β thalassaemia is much less common among cases of leukaemia, MDS and related disorders than an alteration in the ratio of α and β globin chain synthesis.

When acquired thalassaemia occurs, the phenotype is most often that of haemoglobin H disease although acquired α thalassaemia trait [2], β or $\delta\beta$ thalassaemia, increased haemoglobin F and increased or decreased haemoglobin A₂ [3–7] have also been reported. It should be noted that cases described as acquired β or $\delta\beta$ thalassaemia are not as well characterised as acquired haemoglobin H disease and it is less clear that this is a distinct entity. The molecular mechanism, deletion of the α globin gene cluster from one chromosome 16, was identified in a single patient with acquired α thalassaemia trait as a feature of MDS [2].

More than 80 cases of acquired haemoglobin H disease have been described [8–23]. This syndrome has been associated mainly with MDS but also with AML, primary myelofibrosis, atypical chronic myeloid leukaemia and various difficult-to-classify myelodysplastic/myeloproliferative neoplasms. It has been described in a 10-year-old boy with Down syndrome and it was postulated that this represented the onset of MDS [24]. The type of MDS most strongly associated with acquired haemoglobin H disease is refractory anaemia with ring sideroblasts (Fig. 6.1) (myelodysplastic neoplasm with *SF3B1* mutation in the 2022 World Health Organization classification) but some cases have had MDS with low blasts or MDS with increased blasts in this classification. Cases of AML have typically been erythroleukaemia (Fig. 6.2), sometimes with sideroblastic erythropoiesis. Atypical myelodysplastic/myeloproliferative neoplasms have included several cases of refractory anaemia, with or without ring sideroblasts, with coexisting thrombocytosis.

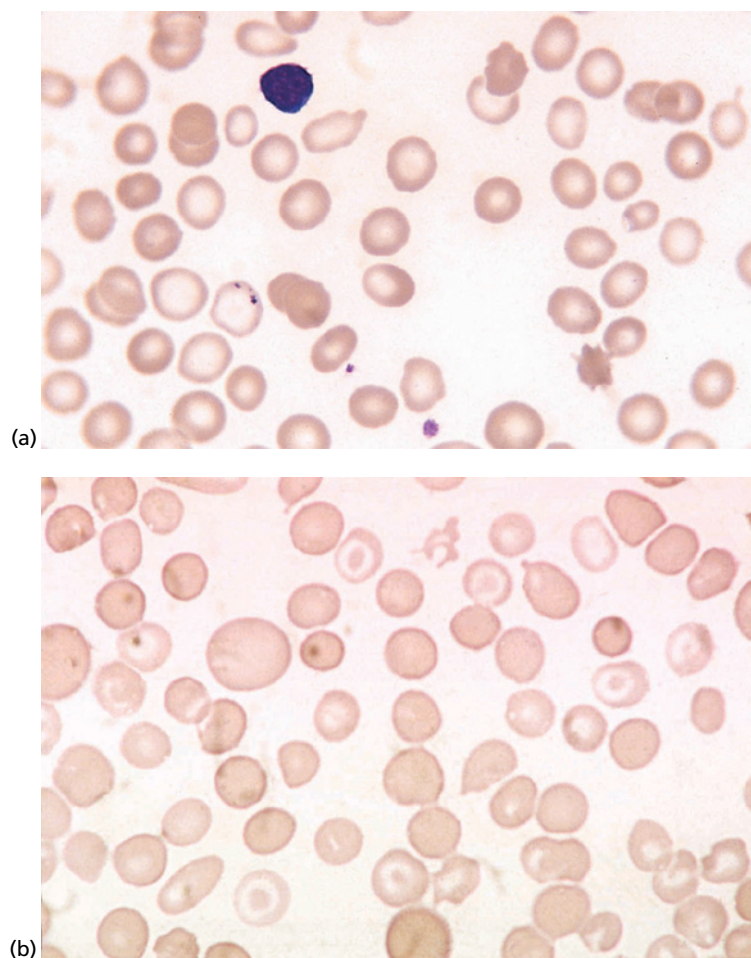


Fig. 6.1 Blood film from a 60-year-old man with acquired haemoglobin H disease associated with myelodysplastic syndrome (MDS) previously characterised as refractory anaemia with ring sideroblasts, now MDS with *SF3B1* mutation. The full blood count (FBC) showed white cell count (WBC) $9.2 \times 10^9/l$, haemoglobin concentration (Hb) 100 g/l, mean cell volume (MCV) 66 fl and platelet count $53 \times 10^9/l$. The haemoglobin H percentage was 9% and the $\alpha:\beta$ chain synthesis was 0.16: (a) dimorphic red cells with one of the hypochromic cells containing several Pappenheimer bodies; (b) dimorphic red cells with normocytic normochromic cells and microcytic target cells. (With thanks to Dr A. Hendrick.) May–Grünwald–Giemsa (MGG) $\times 100$ objective.

One case of acquired haemoglobin H disease has been reported which was associated with what appeared to be acute lymphoblastic leukaemia [18], an unexpected association. Although no immunophenotyping was available, there was strong circumstantial evidence of lymphoid lineage: the cells showed periodic acid–Schiff (PAS)-block positivity and were negative for Sudan black B and myeloperoxidase and the disease remitted on two occasions with vincristine and prednisone. The disappearance of the haemoglobin H with disease remission, on two occasions, provided evidence that the haemoglobin H-containing red cells and the leukaemic blasts belonged to the same clone. If the leukaemia was indeed lymphoid, a leukaemogenic mutation in a pluripotent lymphoid-myeloid stem cell could be postulated.

It has been demonstrated that in acquired haemoglobin H disease, although all four α genes are intact and show a normal pattern of methylation, α chain messenger ribonucleic acid (mRNA) is greatly reduced. In many patients the defect in α chain synthesis is so severe that it is clear that all four α genes are downregulated. Interestingly, one patient with acquired haemoglobin H disease had five α genes, as a result of a constitutional triple α , and despite this had an $\alpha:\beta$ globin chain synthesis ratio of 0.09 [19]. In this patient all five α genes must have been downregulated. Clearly, a *trans*-acting factor was implicated. This was supported by the observation that the ratio of $\alpha 2$ and $\alpha 1$ gene transcripts was normal [20]. The great majority of patients with acquired haemoglobin H disease, more than 90%, have been

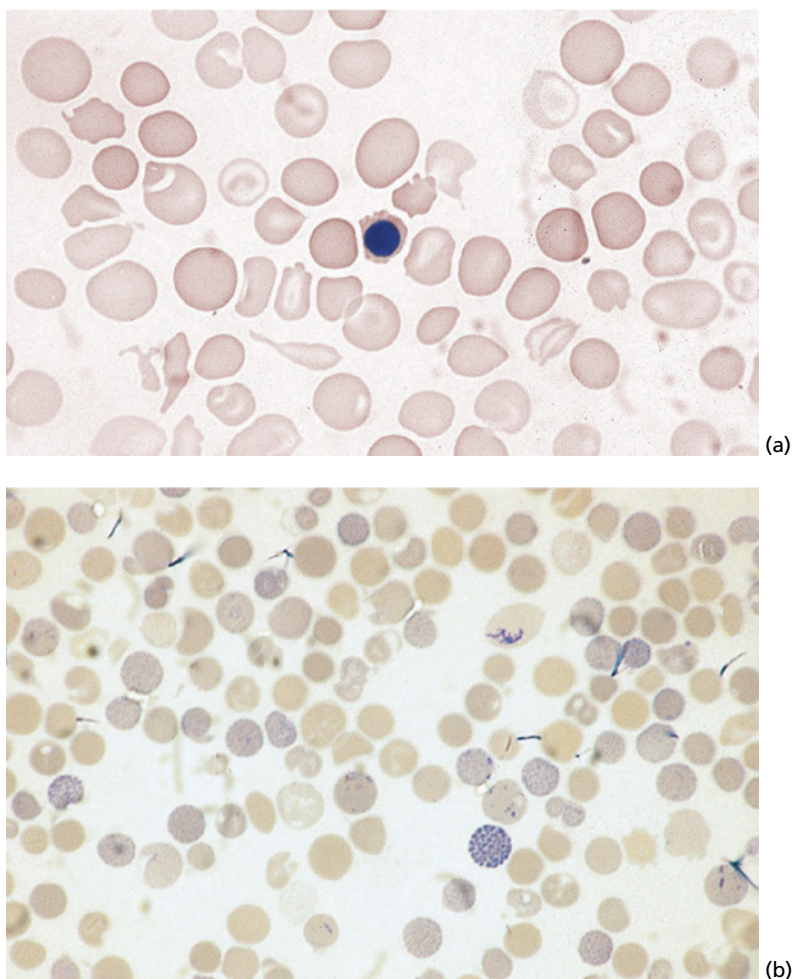


Fig. 6.2 Acquired haemoglobin H disease in a patient with erythroleukaemia: (a) blood film, MGG $\times 100$; and (b) haemoglobin H preparation. Haemoglobin H was 15% of total haemoglobin.

male. The explanation of all these observations was revealed when it was demonstrated that the cause of acquired haemoglobin H disease is an acquired somatic mutation of the *ATRX* gene [22, 25, 26]. A unique case involved chromosome 16 rather than the *ATRX* gene on the X chromosome.

Acquired haemoglobin H disease is associated with variable anaemia and reticulocytosis. Microcytosis is common but not invariable, whereas inherited haemoglobin H disease is invariably associated with microcytosis. The red cells usually show marked anisocytosis, poikilocytosis and hypochromia. The blood film is often dimorphic, a feature that is not seen in the inherited form of the disease (Fig. 6.3). There are three possible explanations for the

dimorphism: (i) the frequency of association of acquired haemoglobin H disease with sideroblastic erythropoiesis; (ii) the possibility that there is coexistence of abnormal clonal cells, showing a defect in α chain synthesis, and surviving normal cells with normal haemoglobin synthesis; and (iii) the possibility that the erythroid precursors with a defect in synthesis of α chain and haemoglobin represent a subclone of the neoplastic population. In occasional cases the α chain synthetic defect leading to haemoglobin H synthesis has either appeared during the course of the illness [12] or has decreased with disease progression [19], supporting the third possibility. The percentage of haemoglobin H varies considerably between cases, from a few percent to as high as 60–65%. Cells containing

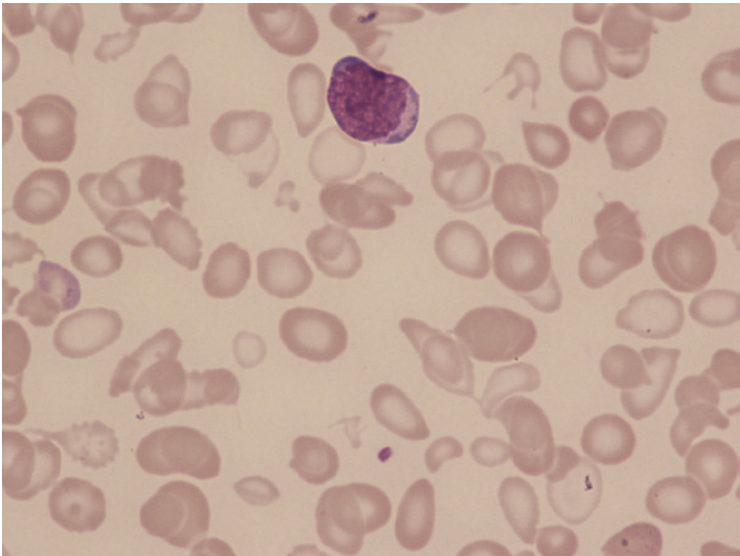


Fig. 6.3 Blood film of a patient with acquired haemoglobin H disease and MDS characterised as myelodysplastic syndrome with excess blasts showing a blast cell, dimorphic red cells and marked anisopoikilocytosis. No ring sideroblasts were observed in the bone marrow aspirate. Haemoglobin H was 4% and numerous inclusions were seen in a haemoglobin H preparation. MGG $\times 100$.

haemoglobin H inclusions vary from a few percent to more than 90%. Cases with an acquired *ATRX* mutation have more severe haemoglobin H disease than is seen in the inherited *ATRX* syndrome, even when the mutation is the same, with a median of 15% haemoglobin H and a median of 30% of cells having H inclusions [22, 26, 27]. Haemoglobin H levels are also often higher than in inherited haemoglobin H disease and the $\alpha:\beta$ chain synthesis ratio is often much lower, varying from 0.05 to 0.66. Some cases have traces of haemoglobin Bart's. When

haemoglobin H constitutes a large percentage of total haemoglobin, the oxygen dissociation curve becomes very abnormal and, because of the hyperbolic dissociation curve of haemoglobin H, symptoms are much more severe than would be expected for the degree of anaemia. The percentage of haemoglobin A_2 is reduced. Uncommonly haemoglobin F is increased [28].

At least seven patients have been reported with acquired thalassaemic conditions resembling β thalassaemia (Table 6.1) [3–6, 29, 30]. Two cases with a low normal or reduced

Table 6.1 Acquired β , $\delta\beta$ and $\gamma\delta\beta$ thalassaemia. (From references [3–6, 29 and 30].)

Sex/ age (years)	Hb (g/l)	MCV (fl)	F %	A_2 %	$\alpha:\beta^*$	$\alpha:\beta+\gamma^*$	$\gamma:\alpha\gamma$	Disease	Reference
M, 27	89	↑	39	0.85	2.2	1.2–1.4	99:1	M6 AML†	[3]
M, 73	65	107	16	1.9	1.85	1.18	0.85:1	RARS	[4]
M, 26	103	80	3	1.3	1.7	1.7	NS	RAEB	[5]
M, 30	78	67	<1	2.4	1.4	NS	NS	CVID	[29]
M, 22	138	75	<1	2.9	1.15	NS	NS	CVID	
F, 3	105	67	77	0.4	2.24	0.45	2.2:1	JMML	[6]
F, 65	97	66	3.1	2.4				MDS	[30]

AML, acute myeloid leukaemia; CVID, common variable immunodeficiency; JMML, juvenile myelomonocytic leukaemia; MDS, myelodysplastic syndrome; RAEB, refractory anaemia with excess of blasts; RARS, refractory anaemia with ring sideroblasts.

* Globin chain synthesis ratios.

† M6 AML (erythroleukaemia) according to the French–American–British classification with preceding aplastic anaemia.

haemoglobin A₂ percentage and an increased haemoglobin F percentage were reported as 'acquired $\delta\beta$ thalassaemia' [3, 4]. The remaining cases were reported as 'acquired β thalassaemia' [5, 6, 29, 30] although none of them had an increased haemoglobin A₂ percentage. Two of these cases had a significantly increased haemoglobin F percentage [5, 6] and are therefore better regarded as acquired $\delta\beta$ thalassaemia. The other two had microcytosis and unbalanced chain synthesis but with no increase in either haemoglobin A₂ or haemoglobin F [29]; a further patient had deletion of the β gene cluster [30] (and could therefore be regarded as having acquired $\gamma\delta\beta$ thalassaemia).

Acquired β , $\delta\beta$ and $\gamma\delta\beta$ thalassaemias have been described in MDS [4, 5], AML (erythroleukaemia) [3], juvenile myelomonocytic leukaemia [6] and common variable immunodeficiency [29]. The phenotype has usually resembled the heterozygous state for these thalassaemic disorders [3–5, 29]. However, a child with juvenile myelomonocytic leukaemia has been described with a phenotype resembling β (or more correctly $\delta\beta$) thalassaemia major [6]. Although it is common for children with this type of leukaemia to have an increased rate of haemoglobin F synthesis with reversion to the usual fetal $\zeta\gamma$: $\alpha\gamma$ ratio, this child differed in also having marked microcytosis and very unbalanced chain synthesis. The phenotype of some cases of acquired β , $\delta\beta$ and $\gamma\delta\beta$ thalassaemia has differed from that of the inherited disorders. Two patients had a significant macrocytosis rather than microcytosis [3, 4] and one had marked reticulocytosis [5].

The leukaemias and MDS can be viewed as acquired genetic disorders affecting a haemopoietic stem cell. It is therefore not surprising that there can be mutations affecting globin chain synthesis. However, although the relevant mRNA is greatly reduced, mutations in globin genes have rarely been detected [5, 6, 20, 27].

Two cases of acquired β thalassaemia and one of acquired α thalassaemia have been reported in patients with common variable immune deficiency [29], another unexpected association. In one patient there was significant anaemia and microcytosis with normal haemoglobin electrophoresis and an α : β chain synthesis ratio of 0.76. X-linked polymorphism analysis

suggested clonal haemopoiesis and lymphopoiesis. It was postulated that there had been damage to a common lymphoid-myeloid stem cell with subsequent clonal expansion.

It is possible for a somatic mutation to interact with a germ line mutation as occurred in a patient with heterozygosity for β^0 thalassaemia who had lost the second β allele in a proportion of cells, leading to the phenotype of β thalassaemia intermedia [31]. This mutation occurred during embryogenesis, rather than as a result of a haematological neoplasm. However, a number of instances have been reported of late onset β thalassaemia intermedia or major as a result of acquired uniparental disomy in patients who had previously manifest β thalassaemia heterozygosity [32, 33].

Increased or decreased haemoglobin F

Increased synthesis of haemoglobin F is relatively common. Decreased synthesis, other than in γ thalassaemia, is rare (or at least is difficult to demonstrate beyond the neonatal period since the percentage of haemoglobin F is normally low). A reduced percentage has been reported in Down syndrome [34] and in a single neonate with a chromosome group C/D translocation [35]. Decreased synthesis of haemoglobin F in the fetal or neonatal period can be viewed as premature switching from γ chain to β chain synthesis, since any deficit in haemoglobin F synthesis is compensated for by haemoglobin A synthesis.

An alteration of haemoglobin F level can result not only from an alteration in the rate of synthesis but also from longer or shorter survival of haemoglobin F-containing cells. For example, in the neonatal period a reduced level of haemoglobin F can result from a haemolytic anaemia that leads to rapid destruction of haemoglobin F-containing cells with new cells that are formed containing a higher proportion of haemoglobin A. There is no alteration in the relative rates of synthesis of β and γ globin chains [36]. The converse is seen later in life when an increased haemoglobin F level in some inherited disorders of globin chain synthesis results from preferential survival of cells that contain more haemoglobin F. This can occur in

sickle cell anaemia, β^+ and β^0 thalassaemia and with unstable β chain variants. However, in acquired conditions increased synthesis is the only known mechanism for a higher haemoglobin F.

The main determinant of haemoglobin F percentage in the neonatal period is post-conceptual age so that premature babies have a higher proportion; the levels are, however, appropriate for the gestational age of the neonate. Post-mature babies have a lower haemoglobin F than babies born at term. Intrauterine hypoxia or growth retardation leads to a higher haemoglobin F in the neonate. On multiple regression analysis, neonatal haemoglobin F level correlates, in addition, with male sex, twin births and maternal cigarette smoking [37]. An association with maternal cigarette smoking was confirmed in a second study [38]. Maternal diabetes mellitus is also associated with a higher level due to higher levels of short-chain fatty acids. It should be noted that although these conditions cause a higher than normal percentage of haemoglobin F at birth, and this is therefore correctly regarded as congenital, this is nevertheless 'acquired' in the sense that it is not genetic but is due to adverse conditions operating during intrauterine life.

A study of sudden infant death syndrome suggested that haemoglobin F percentage was increased, in comparison with levels seen in infants matched for post-conceptual age [39] but this was not confirmed in two subsequent studies [40, 41].

Acquired causes of an increased percentage of haemoglobin F are shown in Table 6.2. [36–38, 42–50] (for inherited causes see Table 3.13).

A rise in haemoglobin F level is seen in 15–20% of pregnant women with levels up to 5% [36]. This is related to an increasing red cell mass and a burst of F-cell production. An increased rate of synthesis of haemoglobin F is also a feature of 'stress erythropoiesis' when there is rapid erythroid expansion and high levels of erythropoietin and glucocorticoids. There is an associated increased in mean cell volume (MCV) and increased expression of i antigen, normally expressed at high levels only in the fetal and neonatal periods. The ratio of $G\gamma:A\gamma$

synthesis in stress erythropoiesis may be that characteristic of fetal life [44]. Stress erythropoiesis may be seen following blood loss or acute haemolysis, during recovery from bone marrow or red cell aplasia and shortly after erythropoietin administration.

Haemoglobin F synthesis is increased in certain leukaemias. This is characteristic of juvenile myelomonocytic leukaemia (Figs 6.4 and 6.5) and is often used as one of the features defining this disorder. The mechanism is unknown but the disease is characterised by the presence of true fetal haemopoiesis, with decreased haemoglobin A_2 , decreased expression of erythrocyte I antigen, increased expression of erythrocyte i antigen and reduced expression of carbonic anhydrase. When the haemoglobin F percentage is increased, the $G\gamma:A\gamma$ ratio is that usually seen in fetal life rather than that usually seen beyond the first few months of life. An increased percentage of haemoglobin F has been associated with a worse prognosis in children with this type of leukaemia [51].

Haemoglobin F levels can be raised pharmacologically (e.g. by administration of short-chain fatty acids, acetylating agents, immunomodulatory drugs, hydroxycarbamide and many cytotoxic agents). Higher levels observed in patients with human immunodeficiency virus (HIV) infection may be related to the administration of zidovudine [52].

Increased or decreased haemoglobin A_2

A low level of haemoglobin A_2 synthesis is physiological in the fetus and neonate.

At other stages of life, a low rate of synthesis can be inherited or acquired. Reduced synthesis of haemoglobin A_2 is relatively common as an acquired disorder, as a result of iron deficiency or impaired delivery of iron to developing erythroid cells. It should be noted that an acquired condition leading to decreased synthesis of haemoglobin A_2 can lower the percentage in a patient with β thalassaemia trait and, in a mild case, could obscure the diagnosis. A low haemoglobin A_2 percentage, with or without an

Table 6.2 Some acquired causes of an increased percentage of haemoglobin F. (From references [36–38, 42–50] and other sources.)

Neonatal period (acquired *in utero*)

Premature babies
Small for gestational age babies
Chronic intrauterine hypoxia
Infants of diabetic mothers [45, 46]
Maternal cigarette smoking [37, 38]

Infancy and childhood

Juvenile myelomonocytic leukaemia
Recovery from transient erythroblastopenia of childhood

Adults or any age

Pregnancy (second trimester when rapid erythroid expansion is occurring)

‘Stress erythropoiesis’

Blood loss or phlebotomy
Acute or severe haemolysis
Recovery from bone marrow failure, e.g. following treatment of acute leukaemia, recovery from severe infection
Recovery from pure red cell aplasia
Recovery from iron deficiency anaemia
Erythropoietin administration.

During regeneration following bone marrow transplantation

Aplastic anaemia, particularly during androgen administration

Pernicious anaemia

Some cases of acute myeloid leukaemia (particularly erythroleukaemia), myelodysplastic syndromes, paroxysmal nocturnal haemoglobinuria and polycythaemia vera

Tumours including hydatidiform mole, carcinoma of the bronchus and breast, ovarian and testicular tumours, gastrointestinal tumours, hepatoma and multiple myeloma [47]

Thyrotoxicosis [48]

Diabetes mellitus (minor increase) [50]

Starvation ketoacidosis [36]

Kala azar [44]

Drug-induced

Hydroxycarbamide administration in sickle cell anaemia or β thalassaemia intermedia

Butyrate administration in sickle cell anaemia

Acyating agents, such as phenylacetate and phenylbutyrate, in sickle cell anaemia

Azacitidine administration in sickle cell anaemia

Immunomodulatory drugs, including thalidomide, pomalidomide and lenalidomide

Erythropoietin

Sodium valproate

increased percentage of haemoglobin F, may be predictive of leukaemic transformation in aplastic anaemia [53].

An increased haemoglobin A₂ percentage is an uncommon acquired abnormality except in the context of HIV infection and its treatment.

Acquired abnormalities leading to an increased or reduced percentage of haemoglobin A₂ are summarised in Table 6.3 [48, 53–63] (for inherited causes of an abnormal haemoglobin A₂ percentage see Tables 3.8 and 3.12).

Increased or decreased glycosylated haemoglobin

Addition of glucose to the N-terminus of the β chain, forming haemoglobin A_{1c}, is a normal post-translational modification of haemoglobin. The concentration is usually 3–4%. Other glycosylated fractions are present at a lower concentration; these include haemoglobins A_{1a} and A_{1b}. A higher concentration of blood glucose in diabetes mellitus leads to an increased percentage of glycosylated

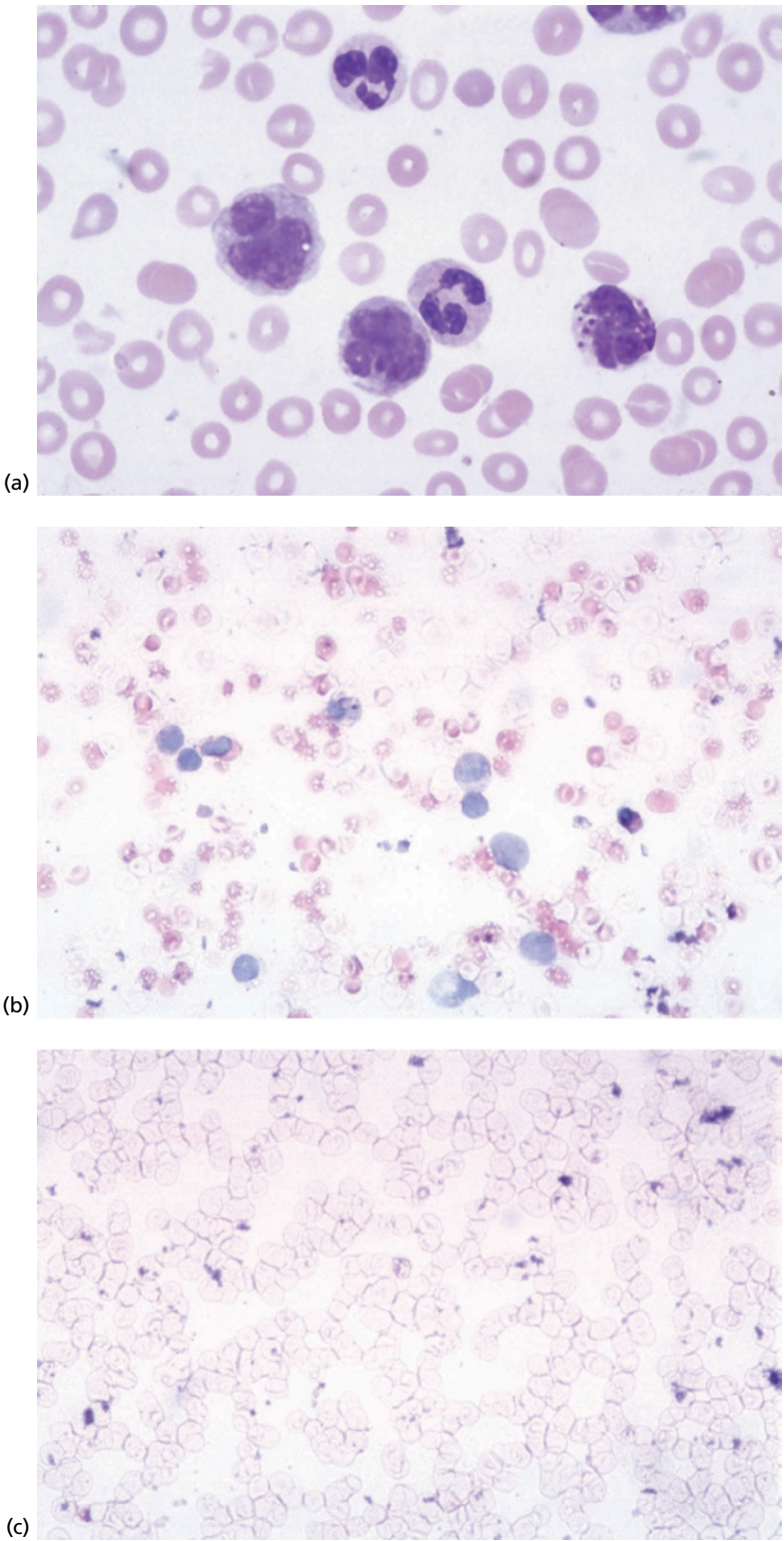


Fig. 6.4 Juvenile myelomonocytic leukaemia showing: (a) blood film MGG $\times 100$; (b) Kleihauer test showing increased F-containing cells ($\times 40$); and (c) negative control for Kleihauer test ($\times 40$). (With thanks to Dr Bridget Wilkins, Winchester.)

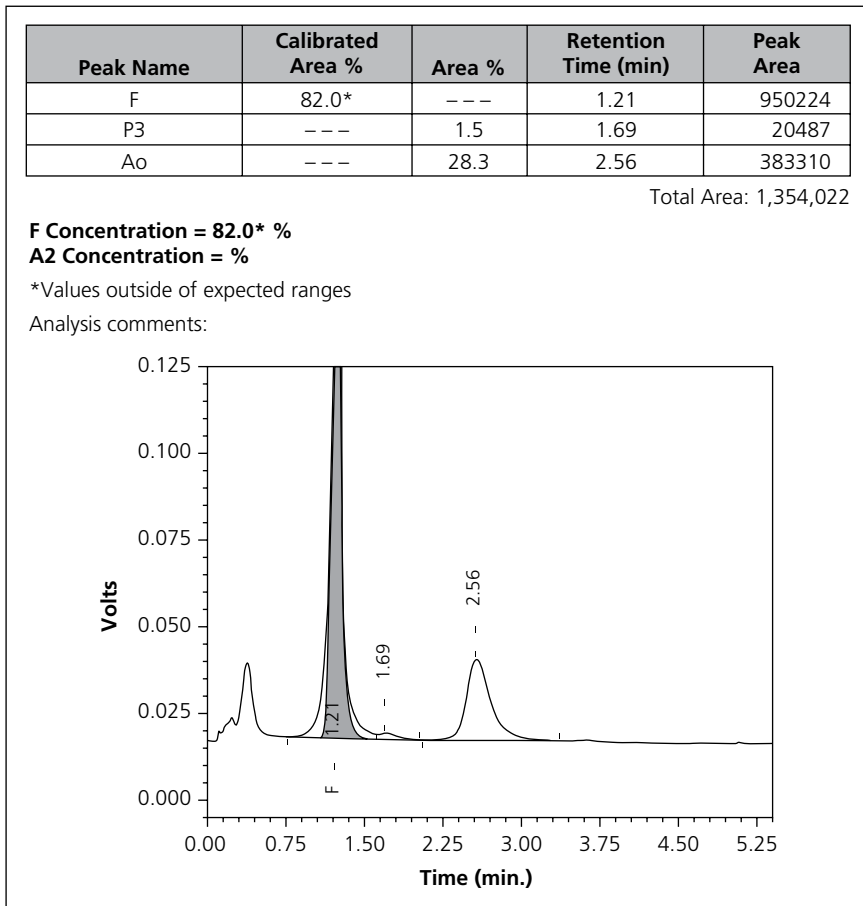


Fig. 6.5 High performance liquid chromatography (HPLC) chromatogram (Bio-Rad Variant II) in juvenile myelomonocytic leukaemia showing, from left to right, post-translationally modified haemoglobin F, haemoglobin F₀ and haemoglobin A. Note the acquired absence of haemoglobin A₂.

haemoglobin (Fig. 6.6). Glycosylation is irreversible but does not have any major effect on haemoglobin function. The oxygen affinity is mildly increased as interaction with 2,3-diphosphoglycerate (2,3-DPG) is impaired [64]. Not only haemoglobin A but also other normal and variant haemoglobins are glycosylated. Glycosylated haemoglobins can sometimes lead to diagnostic confusion. For example, on high performance liquid chromatography (HPLC), a glycosylated haemoglobin may have the same characteristics as another normal or variant haemoglobin; with some systems glycosylated haemoglobin S elutes very close to haemoglobin A. In addition, when glycosylated haemoglobin is

measured for monitoring of diabetes mellitus, the total may be underestimated if haemoglobin A_{1c} is measured but the presence of a glycosylated variant is not appreciated.

Haemoglobin A_{1c} is lower than normal in patients with haemolytic anaemia since glycosylation increases with the average age of the red cell, and haemolysis usually involves the preferential loss of older cells. It is higher than normal in transient erythroblastopenia of childhood [65], indicating the older red cell population. It is significantly increased in iron deficiency anaemia [66]. Other real and artefactual causes of increased or decreased

Table 6.3 Some acquired causes of an increased or decreased percentage of haemoglobin A₂ [48, 53–63].

Increased percentage

Hyperthyroidism [48]
Megaloblastic anaemia consequent on deficiency of vitamin B₁₂ or folic acid (some cases)* [54, 55]
Human immunodeficiency virus (HIV) infection and its treatment with zidovudine [56–59]
Associated with hypertrophic osteoarthropathy [60]
Malaria [61]

Decreased percentage

Iron deficiency
Anaemia of chronic disease [62]
Sideroblastic anaemia [55]
Lead poisoning [55]
Juvenile myelomonocytic leukaemia
Some myelodysplastic syndromes, including acquired haemoglobin H disease
Some cases of acute myeloid leukaemia, particularly erythroleukaemia
Some cases of aplastic anaemia [53]
Hypothyroidism [55]
Associated with chemotherapy-induced increased haemoglobin F synthesis [63]

* But note that folic acid deficiency has been reported to lower the haemoglobin A₂ percentage in individuals with β thalassaemia [54].

haemoglobin A_{1c} percentage are shown in Table 6.4 [65–68].

Carboxyhaemoglobinaemia

Carboxyhaemoglobin is formed when carbon monoxide (CO) combines with haem iron. Carbon monoxide is a product of incomplete combustion of hydrocarbons. Endogenous production of CO as a result of catabolism of haemoglobin (specifically haem), and to a lesser extent myoglobin, is a physiological process so that a low level of carboxyhaemoglobin is detectable in everyone [44, 69]. Healthy non-smokers have around 1% of carboxyhaemoglobin with higher levels (around 5%) being observed in pregnancy and in the presence of haemolytic anaemia [70] or a haematoma [71]. Fetuses and neonates have higher levels than adults [71]. A much higher level of carboxyhaemoglobin in the blood occurs if there is exposure to exogenous CO. Increased levels of carboxyhaemoglobin can result from exposure to:

- car exhaust fumes (deliberate or accidental, including snow-blocked car exhaust pipe [72] – the risk is less when a catalytic converter is fitted);

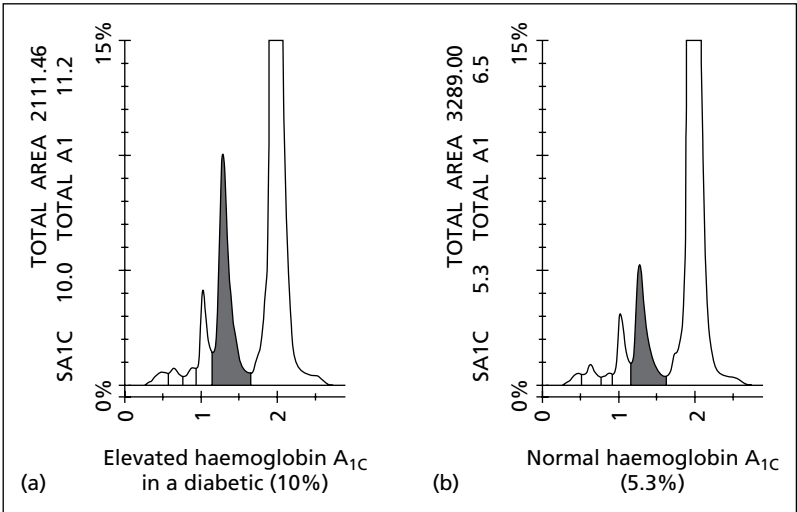


Fig. 6.6 HPLC chromatogram showing: (a) increased haemoglobin A_{1c} in a diabetic patient; and (b) normal haemoglobin A_{1c} in a control sample.

Table 6.4 Causes of real and apparent increased and decreased percentages of haemoglobin A_{1c} [65–68].

	Increased percentage	Decreased percentage
Factitious	Presence of a variant haemoglobin with a retention time overlapping with haemoglobin A _{1c}	Presence of various variant haemoglobins including haemoglobins S, C, Takamatsu, G-Szuhu, Himeyi, O-Padova, Camden, Riyadh, J-Meerut, Sherwood Forest, Manitoba and G-Coushatta [67]
True	Diabetes mellitus Aged population of red cells, e.g. recent onset of pure red cell aplasia or transient erythroblastopenia of childhood [65] Iron deficiency anaemia [66] HIV infection [68] Exchange transfusion*	Shortened red cell life span

HIV, human immunodeficiency virus.

* As a result of glycosylation of red cells during suspension in a glucose solution.

- coal, gas, peat and charcoal fires, water heaters, central heating boilers, heating appliances, power generators, other combustion engines – particularly when ventilation is poor and if a fire intended for outdoor use is used indoors or in a sealed tent;
- inhaled smoke in house fires;
- industrial fumes, including both propane and methane used as fuels and methylene chloride (a common component of paint remover and other solvents, which is metabolised by the liver to CO) [69];
- cigarette smoke and hookah smoking;
- coffee roasting in a confined space [73].

Cigarette smokers often have carboxyhaemoglobin levels of 5–10% but sometimes up to 20%. Higher levels (e.g. 38%) can be seen with hookah smoking [74]. Part of the risk to the fetus of cigarette smoking in pregnancy is attributable to CO [71]. Much higher levels, which may be fatal, are seen in suicide attempts using car exhaust fumes and with accidental exposure to industrial chemicals or the combustion products of poorly ventilated domestic heaters. Carbon monoxide poisoning results from suicide attempts in about a half of instances, is associated with burns in about a quarter of cases and results from other unintentional exposure to carbon monoxide in another quarter [75]. A high concentration of carboxyhaemoglobin gives the blood a cherry-red colour. Symptoms

include headache, lethargy, nausea and vomiting followed by drowsiness, convulsions, coma and death.

The affinity of haemoglobin for CO is 200–250 times as great as its affinity for oxygen. The process of carboxyhaemoglobin formation is slowly reversible. Production of carboxyhaemoglobin moves the oxygen dissociation curve to the left and makes it more hyperbolic. This is because, once two CO molecules are bound to haem groups, the molecule changes to an oxy-conformation, increasing the affinity for oxygen. This means that, in individuals with an increased percentage of carboxyhaemoglobin, the degree of tissue hypoxia is greater than would be expected from the percentage of carboxyhaemoglobin present. The increased oxygen affinity caused by binding to CO is more important than the decreased affinity attributable to 2,3-DPG in explaining the varying $P_{50}O_2$ seen in normal individuals [44]. Carboxyhaemoglobin does not function in oxygen transport and, in addition, oxygen delivery to tissues is impaired as is shown by the altered shape of the dissociation curve. The effects of tissue hypoxia are further aggravated by the binding of CO to myoglobin and to mitochondrial cytochromes [70, 76]. The impaired tissue oxygen delivery in individuals with a chronic increase in the percentage of carboxyhaemoglobin means that there is an erythropoietin-driven increase in the rate of

haemoglobin synthesis and the haemoglobin concentration is increased. This may mean that increased blood viscosity compounds the effects of reduced delivery of oxygen to tissues.

When the concentration of oxygen in the inspired air is lower, the effects of any influences likely to raise the carboxyhaemoglobin concentration are greater; for example, poor combustion of a stove in a sealed tent would have a greater effect at altitude. Conversion of carboxyhaemoglobin to oxyhaemoglobin is accelerated by removal from the source of CO and by ventilation with oxygen, or hyperbaric oxygen treatment. The half-life of carboxyhaemoglobin is 240–320 minutes breathing air, 80–100 minutes breathing 100% oxygen and about 20 minutes with hyperbaric oxygen [76]. Hyperbaric oxygen therapy has been demonstrated to reduce neurological damage [77]. An increased rate of ventilation, for example as a consequence of vigorous exercise or artificial ventilation, lowers the percentage of carboxyhaemoglobin (assuming that the individual has been removed from the source of CO and is breathing inspired air with a low content of CO).

If a pregnant woman is exposed to CO, effects in the fetus are even more severe [69]. Fetal haemoglobin has a greater affinity for CO than haemoglobin A so that the percentage of carboxyhaemoglobin is 10–15% higher in the fetus.

In addition, the half-life of fetal carboxyhaemoglobin F is about twice as long as that of maternal carboxyhaemoglobin so that recovery takes longer [78]. There is an exaggerated leftwards shift of the oxygen dissociation curve with resultant severe tissue hypoxia. There is a similar increased vulnerability to CO poisoning during the first few months of life, when fetal haemoglobin levels remain high, and later in life in individuals with a persistent elevation of fetal haemoglobin [76].

Haemoglobin Zurich is an interesting example of the interaction between inherited and acquired abnormalities of the haemoglobin molecule. It has a greater affinity for CO than does haemoglobin A and, paradoxically, this protects cigarette smokers from haemolysis.

Haemoglobin S polymerisation is also inhibited by carbon monoxide, as carboxyhaemoglobin is largely in the oxy conformation. Carbon monoxide has been shown to prolong red cell survival in sickle cell disease [79], although it has a very narrow therapeutic window, making it difficult to develop as a safe treatment. Oral carbon monoxide releasing molecules are now being developed to treat sickle cell disease with encouraging early results [80].

Carboxyhaemoglobin is detected and measured by spectroscopy (Fig. 6.7). However, it should be noted that the severity of CO poisoning

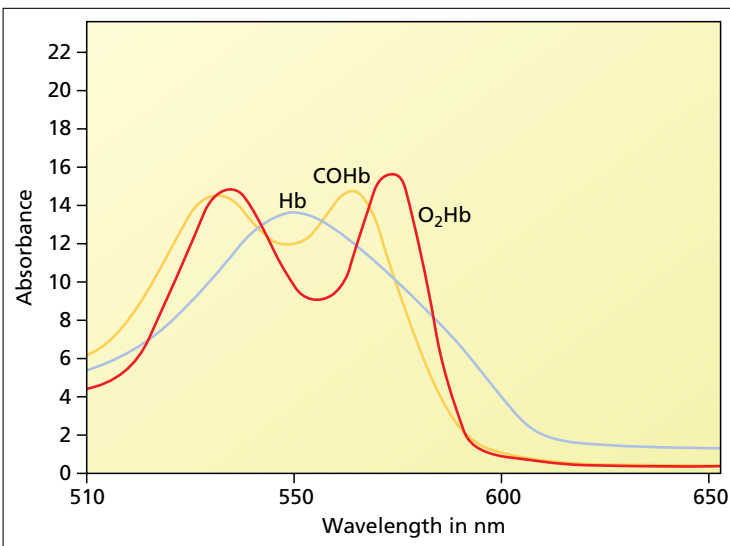


Fig. 6.7 Spectroscopy showing carboxyhaemoglobin (COHb), oxyhaemoglobin (O₂Hb) and deoxyhaemoglobin (Hb).

correlates poorly with the carboxyhaemoglobin percentage and this should not be used for judging the necessity for hyperbaric oxygen therapy [81]. Pulse oximetry is inaccurate in measuring oxyhaemoglobin when carboxyhaemoglobin is present since the technique does not distinguish between carboxyhaemoglobin and oxyhaemoglobin [69]. This is because the wavelengths employed by most pulse oximeters are selected to distinguish between oxygenated and non-oxygenated haemoglobin and not to distinguish oxyhaemoglobin from other form of haemoglobin. A pulse CO-oximeter (or co-oximeter), however, measures the percentages of carboxyhaemoglobin, oxyhaemoglobin and deoxyhaemoglobin. Carboxyhaemoglobin may be seriously underestimated by some spectrophotometers if the patient has been administered hydroxocobalamin as part of emergency management [82].

Methaemoglobinaemia

Methaemoglobin is formed by oxidation of haem iron (i.e. by conversion from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form). Auto-oxidation of haemoglobin occurs, the α chain being oxidised more rapidly than the β chain in an intact molecule. Auto-oxidation is increased by a rise in temperature, increased 2,3-DPG and reduced pH [71]. The rate of production of methaemoglobin is increased by sepsis. Oxidation of

haemoglobin is increased by exposure to exogenous oxidants. Various red cell enzymes convert methaemoglobin back to haemoglobin so that the level is normally less than 1%.

Methaemoglobin does not function in oxygen transport and, in addition, the presence of methaemoglobin leads to a left shift of the oxygen dissociation curve, which further impairs oxygen delivery to tissues; this is because oxidation of some haems in a partly oxidised tetramer favours the oxy conformation of the tetramer. High levels of methaemoglobin are associated with chocolate-coloured blood, cyanosis (or pseudocyanosis) (Figs 6.8–6.10), headache, tachycardia, dyspnoea, tachypnoea and finally coma and death. In patients with glucose-6-phosphate dehydrogenase deficiency, intravascular haemolysis can lead to plasma and serum being brown [83, 84]. The blood film may show evidence of oxidative damage to red cells [85] (Fig. 6.11). Symptoms are considerably greater when an acute rise in the percentage of methaemoglobin occurs than when there is chronic methaemoglobinaemia. A concentration of 0.15–0.2 g/l causes cyanosis (in comparison with the 0.5 g/l of deoxyhaemoglobin that is needed to produce cyanosis). Methaemoglobin is detected by spectroscopy (Fig. 6.12) or by co-oximetry, which permits the detection of carboxy-, met- and sulphhaemoglobins. It should be noted that, as for patients with carboxyhaemoglobinaemia, pulse oximetry and conventional blood gas



Fig. 6.8 The hands of a patient with anaemia and mild methaemoglobinaemia caused by dapsone, in comparison with the hand of a healthy subject. (With thanks to Professor Lawrence Hirst, Brisbane.)



Fig. 6.9 The tongue of a patient with methaemoglobinemia.

analysis are misleading in patients with methaemoglobinemia [86, 87], since methaemoglobin has similar light absorption characteristics to oxyhaemoglobin. Significant amounts of methaemoglobin make pulse oximetry unreliable because the light absorption pattern of methaemoglobin interferes with the calculation of oxygen saturation. Most pulse oximeters give a fixed reading of about 85% in the presence of high levels of methaemoglobin, which does not reflect the underlying oxygen saturation, which may actually be higher or lower [88]. Congenital and acquired causes of methaemoglobinemia are shown in Table 6.5 [44, 71, 83–126]. It is also possible that methaemoglobinemia can be the result of endogenous oxidant production by gut bacteria since two patients have been reported in whom combined met- and sulphhaemoglobinemia responded to oral administration of antibiotics [127] and neonates with diarrhoea and methaemoglobinemia have been reported [128].

Infants are more susceptible to methaemoglobinemia than adults. This is only in part because haemoglobin F is more susceptible to oxidation. It is related more to the reduced level of methaemoglobin reductase at this age.



Fig. 6.10 A bag of blood removed during exchange transfusion (bottom) from a patient with severe methaemoglobinemia induced by an oxidant chemical, showing the chocolate colour conveyed by methaemoglobin. The upper bag, in contrast, is a bag of normal donor blood.

Fig. 6.11 Blood film of a patient with dapsone-induced methaemoglobinaemia showing irregularly contracted cells and keratocytes ('bite cells'). One cell contains a Howell-Jolly body, indicative of functional hyposplenism due to splenic overload during an episode of acute haemolysis. MGG $\times 100$.

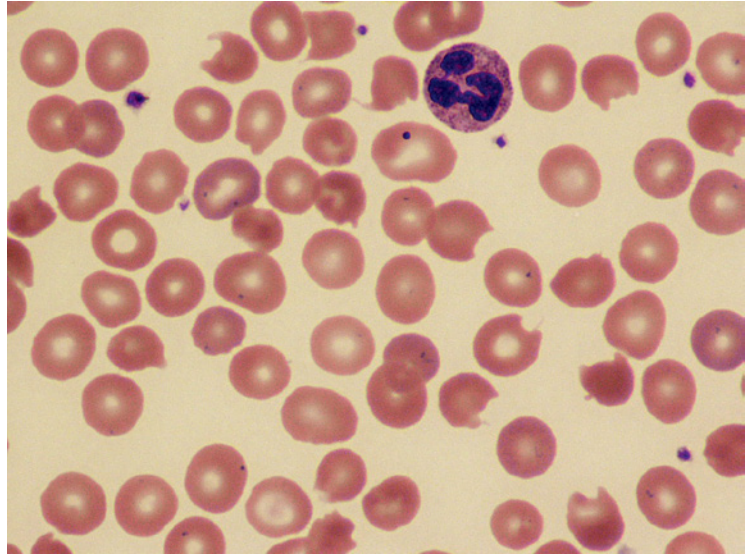
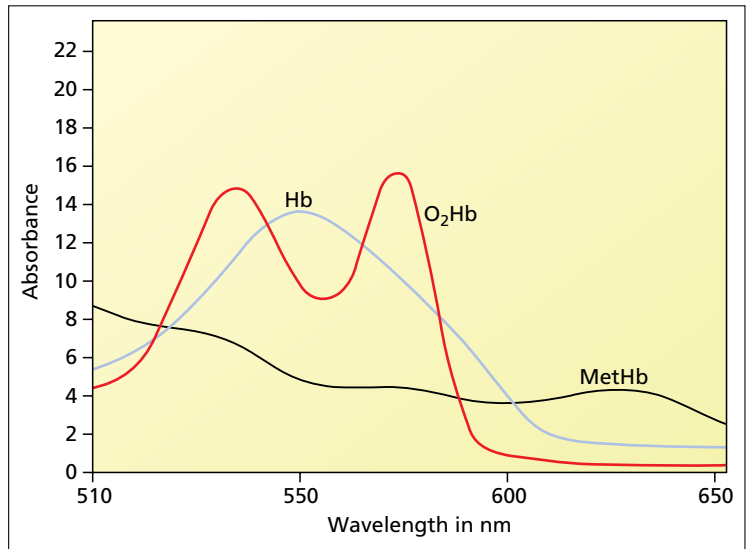


Fig. 6.12 Spectroscopy showing methaemoglobin (MetHb), oxyhaemoglobin (O_2Hb) and deoxyhaemoglobin (Hb).



Very high methaemoglobin levels, greater than 90%, are sometimes seen in association with the use of recreational drugs. These include nitrous oxide (laughing gas), amyl nitrate (poppers) and cocaine adulterated with local anaesthetics or phenacetin.

The congenital methaemoglobinaemias caused by methaemoglobin reductase (NADH-cytochrome b5 reductase) deficiency enter into the differential diagnosis of methaemoglobinaemia. They usually have 10–20% of methaemoglobin.

The reduced oxygen-carrying capacity of the blood leads to secondary polycythaemia. In type I deficiency, methaemoglobinaemia is the only clinical manifestation whereas in type II deficiency, in which tissues other than red cells are affected, there is also neurological impairment and growth retardation. Both have an autosomal recessive inheritance. Individuals with an inherited methaemoglobin reductase deficiency (CYB5R mutation) have an increased susceptibility to oxidant drugs and chemicals.

Table 6.5 Some causes of an increased concentration of methaemoglobin. (Derived from references [44, 71, 83–126] and other sources.)**Inherited**

Haemoglobin M and haemoglobin F-M

As a feature of an unstable haemoglobin

As a feature of haemoglobin E/ β thalassaemia correlating with disease severity and previous splenectomy [89]

Deficiency of NADH-cytochrome b5 reductase

Type I (deficiency of red cells only), AR, *CYB5R3* mutated

Type II (generalised tissue deficiency, neurological abnormalities), AR, *CYB5R3* mutated

Deficiency of cytochrome b5, also known as cytochrome B5A, type IV methaemoglobinaemia, ambiguous genitalia, AR, *CYB5A* mutated

Acquired

Residence at high altitude [90]

Exposure to oxidant drugs and chemicals

Nitrates, nitrites and related compounds

Nitrites such as sodium nitrite*, amyl nitrite† [90], butyl nitrite†, isobutyl nitrite† (therapeutic doses of nitrites may produce up to 5% methaemoglobin; ‘poppers’, used recreationally, are volatile amyl, butyl or isobutyl nitrite)

Nitric oxide (NO) (by inhalation), including nitric oxide administered to neonates with pulmonary hypertension

Nitrates (converted to nitrites by hepatic metabolism), e.g. nitrate-rich diet (particularly in young babies); excessive amount of sodium nitrate used as preservative for sausage meat; Chinese dumplings with a high content of nitrates or containing nitrite-cured meat [91]; nitrate-contaminated well water‡ including when it is used for home dialysis or for reconstituting powdered milk for infants)

Nitroglycerine (intravenously)

Nitroprusside

Nitrobenzenes (shoe and floor polish, paint solvents, agricultural products) [91]

Nitroethane (nail polish remover, propellant, fuel additive) [91]

Nitrofurantoin [91]

Other

3-Aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine®) [93]

Aniline (purchased as a recreational drug) [94] or contained in petrol octane booster or in babies’ nappies [95]

Celecoxib [96]

Chloramine [95]

Clofazimine

Copper sulphate (ingested with suicidal intent)

Dapsone (rarely topical dapsone) [97]

Diarylsulphonylureas [91]

Doxorubicin [91]

Doxycycline (when used in high doses for sclerotherapy) [98]

Favism in G6PD-deficient subjects [99]

Flutamide [100]

Henna

Hypochlorous acid (contained in bleach) [101]

Local or topical anaesthetics§

Topical benzocaine [102] including when used as an oral gel for post-chemotherapy stomatitis [103] and when used for transoesophageal echocardiography [104]

Prilocaine [105]

Procaine

Lidocaine

Table 6.5 *Continued.*

Lysol (50% cresol in linseed oil, potassium hydroxide and water) [106]
Mephedrone (in 'snow' – a recreational drug) [107]
Metoclopramide [90]
Methylene blue (in G6PD-deficient subjects or in high dose) [108]
Naphthalene
Paracetamol (acetaminophen)
Paraquat/monolirurin [95]
Phenacetin (now withdrawn from legal market but sometimes an adulterant in cocaine [95])
Phenazopyridine [109]
Potassium permanganate [110]
Primaquine, chloroquine [91]
Propanil (a herbicide)
Rasburicase [111]
Riluzole (overdose) [112]
Sulphonamides [102] including sulphamethoxazole (constituent of cotrimoxazole)
Unidentified chemicals used by goldsmiths [113]
Electronic cigarettes containing propylene glycol [123]
COVID-19 (SARS-CoV-2 infection) [124]
Food protein-induced enterocolitis in young infants [125]
Neonatal sepsis [126]

G6PD, glucose-6-phosphate dehydrogenase.

* Used for curing meat, as a food preservative, as an insecticide and to inhibit corrosion (may contaminate water in pipes and tanks when it has been used as a corrosion-inhibiting solution) [105]; used in treatment of cyanide poisoning [114]; present in some antifreeze [115].

† Including 'recreational' use.

‡ The risks from well water relate to the application of nitrogenous fertilisers to surrounding farmland [44].

§ Benzocaine and novocaine can be present as adulterants in street heroin [83] and local anaesthetics can be found as adulterants in cocaine [95].

Patients with severe symptoms from methaemoglobinaemia are treated with intravenous methylene blue. In life-threatening situations, exchange transfusion can be employed. Methylene blue is, however, contraindicated in patients with methaemoglobinaemia associated with haemolysis in glucose-6-phosphate dehydrogenase deficiency [92, 93, 129].

Sulphaemoglobinaemia

Sulphaemoglobin is produced by irreversible oxidation and chemical alteration of haemoglobin. The mechanism is probably production of a ferrohaemoglobin–peroxide complex that is sulphated in the presence of hydrogen sulphide [127]. The causes of sulphaemoglobinaemia are summarised in Table 6.6 [44, 130–133]. As already mentioned, it is also likely

Table 6.6 Causes of an increased concentration of sulphaemoglobin [44, 130–133].

Inherited

Autosomal dominant inherited sulphaemoglobinaemia [44]

As a feature of some unstable haemoglobins, e.g. haemoglobin Olmstead [44]

Acquired

Exposure to drugs and chemicals (as for methaemoglobinaemia), but occasionally drug-induced sulphaemoglobinaemia occurs in the absence of methaemoglobinaemia, e.g. with sumatriptan therapy [130]

Sulphur-containing ointments

Occupational exposure to hydrogen sulphide (factory workers, sewage workers, livestock farmers, workers in sulphurous thermal baths)

Escherichia coli septicaemia [131] or urinary tract infection [132]

Intestinal *Morganella morganii* (in a neonate) [133]

that sulphhaemoglobinaemia can result from an abnormal population of gut bacteria since three patients have been reported in whom sulphhaemoglobinaemia, or combined met- and sulphhaemoglobinaemia, responded to oral administration of antibiotics [127]. Abnormal gut flora as a result of constipation has also been suspected as a cause [64]. The drugs that can cause methaemoglobinaemia can also cause sulphhaemoglobinaemia. The most common cause is exposure to drugs such as dapsone and previously phenacetin (sometimes now present as an adulterant in cocaine). Occupational exposure to sulphur-containing compounds can also be responsible. Flutamide, an antiandrogen used for treating carcinoma of the prostate, has been reported to cause sulphhaemoglobinaemia and methaemoglobinaemia [100]. A single case of sulph- and methaemoglobinaemia apparently caused by intravenous all-*trans*-retinoic acid has also been reported [121]. Sulphhaemoglobin lacks cooperativity [64] and does not function in oxygen transport. However, it leads to reduced oxygen affinity of non-sulphinated monomers, ameliorating the effect on tissue oxygen delivery. It has been postulated that sulphhaemoglobinaemia would be much more deleterious in patients with sickle cell disease because favouring of the deoxy-conformation would also favour maintenance of polymerisation [71].

A concentration of 5–16 g/l or above can cause cyanosis (or pseudocyanosis). At very high levels the beneficial effect on oxygen affinity is negated by an extremely right-shifted oxygen dissociation curve. In severe acute sulphhaemoglobinaemia there are neurological effects, pulmonary oedema and death [71].

Sulphhaemoglobin can be detected by spectroscopy (Fig. 6.13) and co-oximetry. Pulse oximetry is inaccurate, the oxygen saturation being overestimated [87].

Methylene blue is ineffective in the treatment of sulphhaemoglobinaemia but, as most patients are asymptomatic, specific treatment is not indicated.

Altered oxygen affinity

Haemoglobin affinity for oxygen may be altered by the administration of drugs given for this purpose, such as voxelotor, which can be given to increase the oxygen affinity of haemoglobin S. Activators of pyruvate kinase, such as mitapivat and etavopivat, also increase haemoglobin oxygen affinity by decreasing 2,3-DPG levels, and are being developed as treatments for sickle cell disease.

A left shift of the oxygen dissociation curve (increased oxygen affinity) was reported in one study of severe SARS-CoV-2 infection

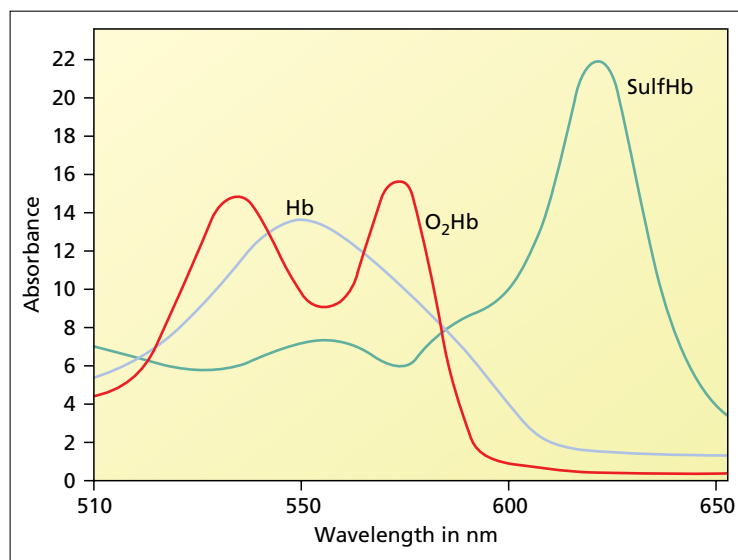


Fig. 6.13 Spectroscopy showing sulphhaemoglobin (sulfHb), oxyhaemoglobin (O₂Hb) and deoxyhaemoglobin (Hb).

(COVID-19) [134] but this was not observed in a second study [135].

Check your knowledge

One to five answers may be correct. Answers to most questions can be either found in this chapter or deduced from information given. Answers are given on page 401.

- 6.1 Cyanosis or pseudocyanosis can be caused by
 - (a) methaemoglobinaemia
 - (b) sulphhaemoglobinaemia
 - (c) carboxyhaemoglobinaemia
 - (d) increased haemoglobin A_{1c}
 - (e) a low affinity haemoglobin
- 6.2 A reduced percentage of haemoglobin A_2 can be caused by
 - (a) haemochromatosis
 - (b) diabetes mellitus
 - (c) anaemia of chronic disease
 - (d) iron deficiency
 - (e) sideroblastic anaemia
- 6.3 An increased percentage of haemoglobin F in a neonate can be caused by
 - (a) intrauterine hypoxia
 - (b) post-mature baby
 - (c) maternal diabetes
 - (d) maternal alcohol intake
 - (e) maternal cigarette smoking
- 6.4 Oxygen affinity of whole blood is increased by
 - (a) a high percentage of haemoglobin F
 - (b) methaemoglobinaemia
 - (c) sulphhaemoglobinaemia
 - (d) a high percentage of haemoglobin A_{1c}
 - (e) carboxyhaemoglobinaemia
- 6.5 An increased percentage of haemoglobin F can be caused by
 - (a) pregnancy
 - (b) the menopause
 - (c) recovery from aplastic anaemia
 - (d) juvenile myelomonocytic leukaemia
 - (e) hydatidiform mole
- 6.6 Acquired haemoglobin H disease is a rare but recognised feature of
 - (a) chronic lymphocytic leukaemia
 - (b) acute myeloid leukaemia
 - (c) myelodysplastic syndromes
 - (d) non-Hodgkin lymphoma
 - (e) congenital sideroblastic anaemia
- 6.7 Carbon monoxide can be derived from
 - (a) metabolism of haemoglobin
 - (b) metabolism of glucose
 - (c) poorly ventilated domestic heaters
 - (d) exhaust fumes of motor vehicles
 - (e) cigarette fumes
- 6.8 An increased percentage of haemoglobin A_{1c} can result from
 - (a) haemolytic anaemia
 - (b) exposure to carbon monoxide
 - (c) recent onset of pure red cell aplasia
 - (d) poorly controlled diabetes mellitus
 - (e) β thalassaemia trait
- 6.9 Carboxyhaemoglobin
 - (a) is formed during the transport of CO_2 from tissues to the lungs
 - (b) is produced by the catabolism of myoglobin
 - (c) is not present in healthy individuals
 - (d) causes the blood to be chocolate-brown in colour
 - (e) cannot be reconverted to oxyhaemoglobin
- 6.10 Methaemoglobinaemia can result from
 - (a) recreational use of nitrites
 - (b) domestic heaters in poorly ventilated rooms
 - (c) occupational exposure to nitrates
 - (d) cigarette smoking
 - (e) an inherited abnormality of haemoglobin structure

- 6.11 The increased percentage of carboxyhaemoglobin in cigarette smokers
 - (a) is greater at increased altitude
 - (b) can lead to an increased haemoglobin concentration
 - (c) can reach 50% of total haemoglobin
 - (d) is reduced by a period of vigorous exercise
 - (e) does not have any disadvantageous effects
- 6.12 Sulphaemoglobinaemia
 - (a) can occur together with methaemoglobinaemia
 - (b) usually causes severe symptoms
 - (c) can be corrected by intravenous injection of methylene blue
 - (d) can be caused by dapsone therapy
 - (e) alters the absorption spectrum of haemoglobin

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Answers to questions

- | | | | | | |
|-----------|-----------|-----------|-----------|------------|------------|
| 6.1 (a) T | 6.3 (a) T | 6.5 (a) T | 6.7 (a) T | 6.9 (a) F | 6.11 (a) T |
| (b) T | (b) F | (b) F | (b) F | (b) T | (b) T |
| (c) F | (c) T | (c) T | (c) T | (c) F | (c) F |
| (d) F | (d) F | (d) T | (d) T | (d) F | (d) T |
| (e) T | (e) T | (e) T | (e) T | (e) F | (e) F |
| 6.2 (a) F | 6.4 (a) T | 6.6 (a) F | 6.8 (a) F | 6.10 (a) T | 6.12 (a) T |
| (b) F | (b) T | (b) T | (b) F | (b) F | (b) F |
| (c) T | (c) F | (c) T | (c) T | (c) T | (c) F |
| (d) T | (d) T | (d) F | (d) T | (d) F | (d) T |
| (e) T | (e) T | (e) F | (e) F | (e) T | (e) T |

7 Organisation of a haemoglobinopathy diagnostic service

General principles

The organisation of a haemoglobinopathy service depends on the ethnic mix of the population served and also on whether the service is hospital based or a regional scheme, whether antenatal and neonatal screening is required, whether children are included in those tested and on the resources available. It is essential that laboratories have clearly defined written protocols that are followed. These will differ, depending on the patient population, the nature of the service that is required and the technology employed. Protocols will differ according to the primary laboratory technique used, whether this be cellulose acetate electrophoresis, high performance liquid chromatography (HPLC), capillary electrophoresis or isoelectric focusing (IEF).

It is important to remember that in a diagnostic laboratory, the identification of a variant haemoglobin is often presumptive rather than definitive and that it is not possible, or indeed clinically necessary, to identify every variant haemoglobin detected. The protocols that are followed should ensure that the great majority of clinically relevant disorders of haemoglobin synthesis are detected. It is not realistic to hope that *all* such abnormalities will be detected. For example, in antenatal screening, silent β thalassaemia will generally be missed. Laboratory tests for haemoglobinopathies should not be performed in a knowledge vacuum. It is essential to know the age of the patient, the ethnic origin, the red cell indices and the clinical features, if any. Legal restraints on documenting

ethnic origin, as in France [1], can make provision of a rational service more difficult. It is essential to know if the patient has been transfused in the recent past. Only with this information will the testing be clinically relevant and appropriately interpreted.

This chapter will discuss mainly the provision of a hospital-based service in a multiethnic community in which the population to be tested includes neonates and children and also pregnant women and their partners, but testing in a resource-poor setting and point-of-care testing will also be considered. In laboratories serving populations of a single ethnic origin, the laboratory procedures will be adapted to local requirements.

All abnormal results of haemoglobinopathy testing should be conveyed to the patient or, in the case of a child, to the parents or guardian, in written form together with an appropriate explanation. To avoid unnecessary repeat testing, it can also be useful to give the patient written confirmation of significant negative results, for example that haemoglobin S is not present. Haemoglobinopathy cards are a useful way to give patients documentation of results, and patient-held or shared electronic records are increasingly used. These should give the patient's name, the date of testing, the test results and, if necessary, an interpretation. It can also be useful to include the haemoglobin concentration (Hb) and the mean cell haemoglobin (MCH) or the mean cell volume (MCV). In the case of normal results in patients with microcytosis from ethnic groups in which α thalassaemia trait occurs, it is

useful to add a statement 'This test does not exclude α thalassaemia trait'.

UK guidelines for screening and diagnosis are available [2].

Antenatal and preconceptual haemoglobinopathy/thalassaemia screening and fetal diagnosis

Antenatal and preconceptual testing

In most countries the aim of antenatal haemoglobinopathy screening is to detect inherited abnormalities in the parents that might lead to disease in the fetus, allowing them to prepare for the birth of a child with significant health problems; in many countries, including the UK, it is also possible to consider a termination of pregnancy if it is predicted that the baby would have a severe condition, incompatible with an independent life. In some instances, specifically when haemoglobin Bart's hydrops fetalis is predicted in the fetus, continuing the pregnancy may also put the mother's own health and even life at risk. In countries where termination of pregnancy is unacceptable or restricted, different approaches are taken to reducing the risk of the birth of an unexpected or unwanted baby with a significant haemoglobinopathy [3].

In some countries with a high prevalence of disorders of globin chain synthesis, screening is usually performed before pregnancy is undertaken. In countries with adequate resources, this can be achieved by population screening, as is carried out, for example, in Cyprus and, more recently, in Saudi Arabia and the United Arab Emirates [4], where screening must be carried out before a marriage certificate is issued. In high prevalence areas, screening of adolescents can also be effective [5]. In some countries marriage is strongly discouraged and marriage licences are refused if both partners are found to have β thalassaemia heterozygosity [6]. In others, most affected couples marry but prenatal diagnosis and termination of an affected pregnancy is often chosen [7]. The percentage of couples who separate when both are found to be carriers varies greatly between countries, from less than 5% to approaching 60%.

In countries with fewer economic resources it may be possible to make testing cost-effective by targeting individuals most in need of testing. For example, in countries such as Pakistan where consanguineous marriages are common and specific disorders of globin chain synthesis such as β thalassaemia tend to segregate in families, screening can be targeted on extended families with an index case [8].

In many countries screening is commonly undertaken only when the potential mother is already pregnant. Tests must therefore be performed rapidly in order to permit testing of the partner and offering of fetal diagnosis and termination of pregnancy, when appropriate, while the pregnancy is still in its early stages. The UK aim is to complete screening of the mother by 10 weeks gestation, to be able to offer prenatal diagnosis to couples by 12 weeks and to carry out prenatal diagnosis by 12 weeks plus six days. Screening should be repeated in subsequent pregnancies. Invasive fetal diagnosis carries a small risk (about 1%) of inducing miscarriage of a fetus, irrespective of its haemoglobinopathy status. For this reason such fetal diagnosis should generally only be undertaken when the potential parents have been fully counselled about the risks, and want to proceed either because they want to prepare for the birth of a baby with a significant haemoglobinopathy or because they are considering termination of pregnancy. Counselling of parents should be based on giving very full information on the likely outcome of pregnancy and the likely severity of any fetal disease. It may be useful for potential parents to be referred to relevant patient support groups. Counselling should be non-directive.

The conditions that should be predicted in a fetus are shown in Table 7.1 and the abnormalities that should therefore be detected in the mother in Table 7.2. It follows that when one of these abnormalities is detected in the mother, appropriate testing of the potential father should follow. Table 7.3 shows conditions that are generally mild and for which prediction is not generally considered necessary. Tables 7.1 and 7.3 represent the consensus view in the UK [9] but there are some areas of controversy,

Table 7.1 Haemoglobinopathies of such severity that the likelihood of their occurrence in a fetus should be predicted.

Haemoglobin Bart's hydrops fetalis
β thalassaemia major and intermedia including that resulting from β thalassaemia/haemoglobin E compound heterozygosity
Sickle cell disease
Sickle cell anaemia
Sickle cell/haemoglobin C disease
Sickle cell/ β thalassaemia
Sickle cell/ $\delta\beta$ thalassaemia
Sickle cell/haemoglobin Lepore
Sickle cell/haemoglobin D-Punjab
Sickle cell/haemoglobin O-Arab
Sickle cell/haemoglobin E

Table 7.2 Disorders of globin chain synthesis that should be detected in prospective parents in order to predict the occurrence of severe disease in offspring.*

α^0 thalassaemia trait or haemoglobin H disease
β thalassaemia trait
$\delta\beta$ thalassaemia trait
Haemoglobin Lepore
Haemoglobin E
Haemoglobin S
Haemoglobin C
Haemoglobin D-Punjab
Haemoglobin O-Arab
Unstable haemoglobins

* Most cases of thalassaemia intermedia and sickle cell disease will already have been diagnosed but occasionally those with quite mild disease will be detected only during pregnancy. UK guidelines suggest hereditary persistence of fetal haemoglobin should also be detected.

Table 7.3 Less severe haemoglobinopathies for which the prediction of the condition in a fetus is not usually considered essential.

Haemoglobin H disease
Mild sickling conditions
Sickle cell/compound heterozygosity with haemoglobin variants other than those listed in Table 7.1
Sickle cell/deletional hereditary persistence of fetal haemoglobin compound heterozygosity
Haemoglobin E homozygosity
Haemoglobin C homozygosity

and practice varies from country to country. Severe haemoglobin H disease is one such area. In countries or ethnic groups where there is a high prevalence of both α^0 and $\alpha^T\alpha$ or other α^T thalassaemia heterozygosity, prediction of haemoglobin H disease can be attempted. However, it should be noted that haemoglobin H disease is often mild and this diagnosis would not generally be considered an indication for termination of pregnancy. The prediction of β thalassaemia intermedia is also a difficult area since this condition varies greatly in severity and the severity of the phenotype associated with a specific genotype is not predictable. This uncertainty must be conveyed to prospective parents. Tables 7.1 and 7.3 are not exhaustive but cover the great majority of likely clinical situations. Parents with rare abnormalities, for example an unstable haemoglobin, need to be assessed individually.

In any antenatal screening programme, it should be remembered that genetic testing causes anxiety, particularly if a woman is already pregnant. In addition, the cost of genetic testing may be considerable and no health service has unlimited resources. This must always be borne in mind when drawing up protocols. It may not be justifiable to test a large number of couples for rare disorders in order to attempt to identify a very small proportion of patients with a significant abnormality, and the design of any screening programme will depend greatly on the prevalence of the targeted conditions in that population. Screening for α^0 thalassaemia provides an example of where zeal should be tempered by consideration of what is reasonable. This condition does occur in the native British, African Caribbeans and Indians but it is very uncommon in all these ethnic groups. Both African Caribbeans and Indians have a high percentage of α^T thalassaemia and screening the large number of individuals with microcytosis that is likely to be attributable to heterozygosity or homozygosity for α^T thalassaemia in the hope of identifying rare individuals with α^0 thalassaemia trait is not usually considered justifiable. Individual circumstances may dictate that certain couples are tested; for example, if there is consanguinity, if they are

native British originating in Lancashire or Cheshire or if they are of Caribbean origin and may have Chinese ancestry.

In antenatal screening, if one partner is found to have β thalassaemia heterozygosity and the other to have α^0 heterozygosity (or haemoglobin H disease), the partner with β thalassaemia heterozygosity should be specifically tested using DNA analysis to exclude coexisting α^0 thalassaemia. The coexistence of the two has been found in 8–9% of Hong Kong Chinese, 4% of Chinese in Guangdong province of China and 8% of Chinese in Malaysia [10]. The same applies to haemoglobin E heterozygotes; the possibility of coexisting α^0 thalassaemia must be considered (and will be particularly suspected if the haemoglobin E percentage is lower than otherwise expected). It should also be noted that red cell indices can be misleading in homozygosity for haemoglobin Constant Spring. In a series of 20 patients, the MCH was not below 26 pg in five subjects and in three it was not below 25 pg [11]; the MCV was never below 80 fl. If screening for haemoglobin Quong Sze is to be done (for prediction of non-deletional haemoglobin H disease), an MCH of less than 27 pg is an effective threshold whereas screening by means of the MCV is ineffective [12].

Uptake of antenatal diagnosis is dependent on cultural factors and on the severity of the condition that is predicted. In an audit of prenatal diagnosis for haemoglobinopathies in the UK in 1998, the uptake was 90% among Cypriots, 41% rising to 65% among Indians, 26% among Pakistanis and 9% among Bangladeshis [13]. Uptake is also higher if prenatal diagnosis is offered earlier in pregnancy and in high prevalence areas of the UK, possibly because of the availability of well-informed doctors and counsellors. In general, uptake is higher when β thalassaemia major is predicted than when sickle cell disease is predicted. Uptake is almost universal when haemoglobin Bart's hydrops fetalis is predicted, although it should be noted that occasional parents are choosing diagnosis followed by intrauterine transfusion rather than termination of pregnancy.

A protocol based on HPLC or capillary electrophoresis for the identification of variant

haemoglobins and β thalassaemia trait, applicable both to universal antenatal screening and in other circumstances, is shown in Fig. 7.1 and a similar protocol applicable when cellulose acetate electrophoresis is the primary diagnostic tool in Fig. 7.2. In England, universal screening, in which blood from every pregnant woman is tested specifically for β thalassaemia and haemoglobin variants, is applied in high prevalence areas (screening tests positive in $\geq 2\%$ of antenatal population) with a selective policy being applied in low prevalence areas (screening tests positive in $< 1\%$); some discretion is applicable between these cut-off points. Fig. 7.3 shows an algorithm applicable in a low prevalence area. The action limits suggested for the diagnosis of β thalassaemia trait have been validated [14, 15].

In the UK, deoxyribonucleic acid (DNA) sequencing of both α and β globin genes is now employed for definitive antenatal screening. The cut-off point adopted in the UK for α^0 thalassaemia screening is an MCH of less than 25 pg. A study in Hong Kong indicated that about 1.8% of individuals with $--^{SEA}/(2/110)$ would be missed by this strategy [16]; a cut-off point of 27 pg for the MCH or 80 fl for the MCV is therefore preferred in Hong Kong. In countries with a high prevalence and heterogeneity of thalassaemias and haemoglobinopathies and with limited resources, other screening policies are appropriate (see later).

When testing of a pregnant woman reveals a potentially significant condition it is useful to issue, with the test result, a proforma that can be followed by antenatal clinic staff during the further management of the patient. This helps to ensure correct patient management and can also be used for audit purposes.

In antenatal screening and testing, it is essential to know if a conception is the result of use of a donor egg or a donor sperm. Thalassaemia major, haemoglobin S/ β thalassaemia and haemoglobin H disease have all resulted from the use of donor eggs or sperm. It is also important to know if either partner is of unknown ethnic origin, for example, because of adoption. In women who become pregnant despite having had an allogeneic stem cell transplant, it is the genetic characteristics that are important; the

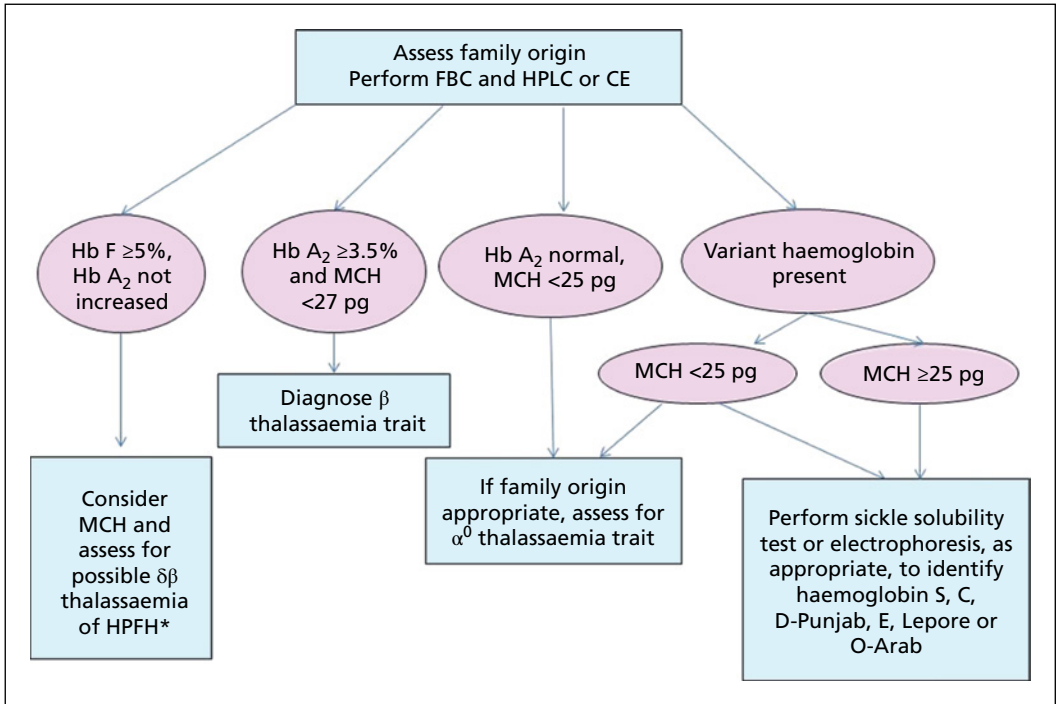


Fig. 7.1 Flow chart for universal antenatal screening for variant haemoglobins and α , β and $\delta\beta$ thalassaemias in a high prevalence area or country, using high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) as the primary method. FBC, full blood count; Hb A₂, haemoglobin A₂; Hb F, haemoglobin F; HPFH, hereditary persistence of fetal haemoglobin; MCH, mean cell haemoglobin. *Hb F $\geq 5\%$ and $< 10\%$ more likely to be $\delta\beta$ thalassaemia, Hb F $\geq 10\%$ more likely to be HPFH.

usual haemoglobinopathy screening result will be misleading.

In countries that have regulations governing DNA analysis these should be followed if testing is DNA based. It is particularly important that very full information about the implications of testing is given when family studies are to be undertaken since these sometimes reveal non-paternity. This is necessary for protein-based testing as well as for DNA-based testing.

Problems and pitfalls in antenatal screening

There are numerous problems and pitfalls in antenatal screening. These include: (i) late booking for antenatal care; (ii) non-availability of partner; (iii) lack of knowledge of ethnic origin; (iv) failure to assess sperm and ovum donors for significant carrier states or non-disclosure of assisted reproduction to the antenatal clinic; (v)

failure to diagnose β thalassaemia heterozygosity with a normal haemoglobin A₂ because of coexisting δ thalassaemia or a δ chain variant, in the latter instance due to failure to sum the percentages of A₂ and the A₂ variant; (vi) failure to consider the possibility of α^0 thalassaemia in individuals with β thalassaemia heterozygosity or haemoglobin E heterozygosity or homozygosity; (vii) failure to predict β thalassaemia intermedia because of silent or near silent thalassaemia; (viii) failure to test for α^0 thalassaemia when the red cell indices are normal because of coinheritance of α^0 thalassaemia and quadruple α [17]. If the mother is found to be a carrier of a significant condition but the partner is not available for testing, it is still possible to proceed with prenatal diagnosis, although the mother should be counselled that the diagnosis may be less accurate. An extra problem that must be avoided in antenatal screening is

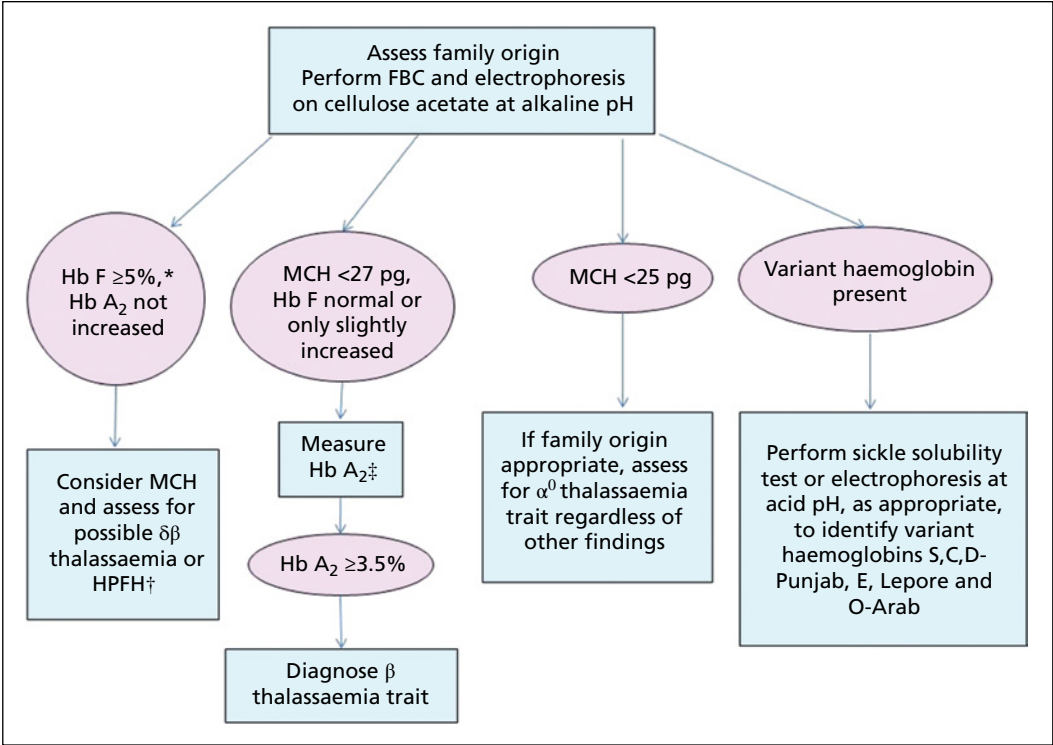


Fig. 7.2 Flow chart for universal antenatal screening for variant haemoglobins and α , β and $\delta\beta$ thalassaemias, in a high prevalence area or country, using cellulose acetate electrophoresis as the primary method. * An apparent increase can be confirmed by a two-minute alkali denaturation test. † Hb F $\geq 5\%$ but $<10\%$ is more likely to be $\delta\beta$ thalassaemia; Hb F $\geq 10\%$ is more likely to be HPFH. In the UK antenatal screening programme, the action value for haemoglobin F is 10% if the MCH is 27 pg or higher and 5% if the MCH is less than 27 pg. ‡ Haemoglobin A₂ is quantified by elution.

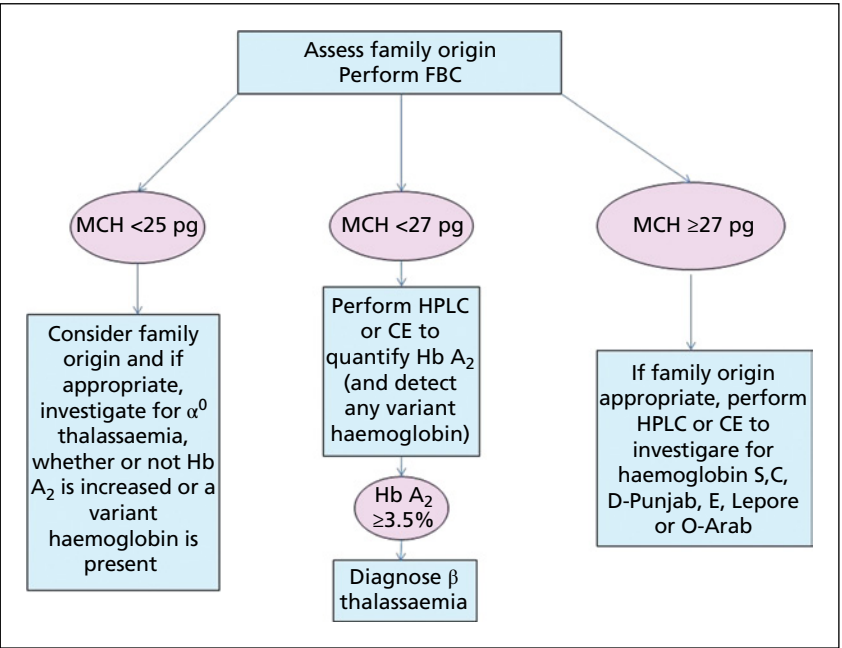


Fig. 7.3 Flow chart for selective antenatal testing in a low prevalence area or country.

stigmatisation of those who are found to be carriers of a significant haemoglobinopathy.

A low percentage of at-risk couples will not be identified despite guidelines being followed, which is inevitable in any screening programme. A relevant study of 967 Indian couples considered, for various reasons, to have a genetic risk of having a child with β thalassaemia major found 24 individuals (2.5%) with a haemoglobin A_2 of less than 3.5% to have a β thalassaemia mutation; the MCH was less than 27 pg in 20 of 24 cases with the MCV being less sensitive (reduced in 17/24) [18]. A population-based study in Saudi Arabia similarly found seven of 96 individuals (18%) with a β thalassaemia mutation to have a haemoglobin A_2 of less than 3%; the MCH was less than 27 pg in six of seven and the MCV was less than 80 fl in three of seven [19].

Fetal diagnosis

Fetal diagnosis can be carried out by chorionic villus sampling (from 11 weeks, usually at 11–14 weeks) or on cells obtained by amniocentesis (from 15 weeks, usually at 15–20 weeks). When amniocentesis is used, back-up cells are cultured in case not enough DNA is obtained; their growth takes 10–14 days so that there may be delay in diagnosis. The aim of the UK scheme is to achieve fetal diagnosis by 12 weeks plus six days of gestation.

Analysis of cell-free DNA in maternal serum has potential for the diagnosis of monogenic disease such as sickle cell anaemia and sickle cell/haemoglobin C disease without the increased risk of miscarriage associated with invasive techniques. In one study, sensitivity was 94% and specificity 88% so that invasive fetal diagnosis was considered necessary to confirm sickle cell anaemia [20]. In another study, specificity and sensitivity were both 100% after exclusion of 4/64 inconclusive results [21].

It may be possible to avoid an invasive test to exclude haemoglobin Bart's hydrops fetalis by use of ultrasound assessment of the fetus. If the middle cerebral artery shows a normal peak systolic velocity then the fetus is not significantly anaemic and hydrops can be excluded.

Assisted reproduction technology with pre-implantation diagnosis of an embryo is also possible when termination of pregnancy would not be acceptable.

Problems and pitfalls of fetal diagnosis

Problems in fetal diagnosis include: (i) those arising from late booking at an antenatal clinic and delayed diagnosis; (ii) miscarriage as a result of the diagnostic procedure; and (iii) illegality of termination of pregnancy in some jurisdictions, sometimes resulting in pregnant women travelling to another country or another state for a termination.

Neonatal screening

In most countries the purpose of neonatal screening is principally the detection of sickle cell disease, since early diagnosis has been shown to reduce mortality during infancy and early childhood, mainly by allowing penicillin prophylaxis to start early, at 2–3 months of age. However, most cases of β thalassaemia major can also be recognised or suspected; for example, between 2010 and 2016 the screening programme in England led to the detection of 113 cases of thalassaemia in addition to 1315 cases of sickle cell disease [22], based on follow-up being offered if haemoglobin A was 1.5% or less. Different aims may be appropriate in countries without a significant incidence of sickle cell disease; in Thailand a neonatal screening programme, aiming to detect $\beta^0\beta^0$ thalassaemia, $\beta^0\beta^E$ and haemoglobin H disease, was considered to have potential to not only enhance the care of babies but also to improve the antenatal screening programme [23].

Neonatal screening can be performed on a cord blood sample (taken by syringe and needle or evacuated container and needle from an umbilical cord vessel after carefully wiping any maternal blood from the surface of the cord), an anticoagulated capillary blood sample, a dried spot of capillary blood (e.g. on a Guthrie card) or, in sick hospitalised babies, a venous sample (Table 7.4). Cord blood should not be squeezed from the end of the cut cord because of the risk

Table 7.4 Methods applicable to neonatal haemoglobinopathy diagnosis.

Method of obtaining blood	Advantages and disadvantages
By needle and syringe from cord vessel	Contamination with maternal blood can occur if technique is not meticulous; inadvertently obtaining a sample after a baby has been transfused is generally avoided; there may be more certainty of testing every neonate if a cord blood sample is used
Blood sample direct from baby, usually venous	Liquid blood sample with low risk of contamination with maternal blood. Potentially difficult to collect, painful for the baby and stressful for parents
Heel-prick sample into a heparinised capillary tube	Transport and labelling of the sample are more difficult but the sample does not suffer the dilution that occurs when a dried blood spot is eluted; contamination by maternal blood is avoided but it is important to avoid inadvertently taking a sample after a blood transfusion has been given
Heel-prick sample blotted on to filter paper and dried	Transport and labelling are easy but dried samples are more likely to be denatured giving blurred bands or peaks; contamination by maternal blood is avoided but it is important to avoid inadvertently taking a sample after a blood transfusion has been given; the blood sample can be obtained at the same time as sampling for metabolic testing, e.g. for phenylketonuria, i.e. at day 5 (or earlier if the baby is about to be transfused)
Method of detecting variant haemoglobins	Advantages and disadvantages
High performance liquid chromatography	Very sensitive technique; variant haemoglobins are quantified
Isoelectric focusing	Very sensitive technique
Capillary electrophoresis	Sensitive technique
Cellulose acetate electrophoresis	An eluate from a Guthrie spot may be too dilute for this technique; less sensitive to low concentrations of a normal or a variant haemoglobin
Tandem mass spectrometry	Applicable only in specialised centres
Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) NeoSickle®	A potentially applicable laboratory-based method
DNA analysis of blood	Definitive diagnosis of most haemoglobinopathies, including α and β thalassaemia, with potential to test for other conditions such as G6PD deficiency. More expensive technique with slower turn-around time

of contamination with maternal blood. A capillary sample obtained by heel prick, blotted on to filter paper and permitted to dry, is usually most convenient. Often one blood spot on a Guthrie card (used for diagnosis of congenital metabolic diseases and, in some African countries, for screening for human immunodeficiency virus infection) is used for this purpose.

When such a dried blood spot is used, a circle of blood-impregnated filter paper is punched out and the haemoglobin is eluted. A Guthrie spot can also be used for DNA analysis. This is useful if a baby has been transfused and greatly reduces the number of babies that have to be recalled for repeat blood sampling. The presence of an adult level of haemoglobin A₂ for

example above 2%, can be a clue to the laboratory that a baby has been transfused.

Universal neonatal screening has been recommended when more than 15% of neonates are born to ethnic minority mothers [24]. In the UK there is now universal neonatal screening for sickle cell disease, using a dried blood spot taken ideally at day 5 and no later than day 8, the day of birth being day 0 [25–27]. UK screening aims to detect the various forms of sickle cell disease and also haemoglobin S/unknown variant and suspected S/hereditary persistence of fetal haemoglobin (HPFH). In addition, results are reported if there is haemoglobin C only, D only, E only or CD, CE or DE. Heterozygotes for these variants and for O-Arab can be reported. Suspected β thalassaemia is reported and is further investigated. Universal screening is also the practice in the Netherlands and Spain; Belgium has universal screening in three regional centres [28] and France has targeted screening in metropolitan France and universal screening in overseas territories [27]. Sickle cell disease is also the target of newborn screening in Greece. Universal screening is US policy. In some circumstances considerations of cost may dictate selective screening. Screening can be performed before the baby leaves hospital, in a hospital-based scheme, or together with screening for inherited metabolic defects, in a community-based scheme. Neonatal screening programmes for sickle cell disease are starting to be developed in many African countries, with regional programmes based in parts of many countries, including Benin, Burkina Faso, Ghana, Cameroon, Democratic Republic of the Congo, Gabon, Guinea, Kenya, Liberia, Mali, Niger, Nigeria, Senegal, Tanzania, Uganda and Zambia. Similarly, many states in India have established neonatal screening for sickle cell disease. In most of these low- and middle-income countries, screening is increasingly based on using point-of-care testing devices. In high-income settings, the most suitable techniques are those that are sensitive and can be performed with small blood samples (e.g. HPLC, capillary electrophoresis, IEF and tandem mass spectrometry) [22, 29–32]. A suitable protocol is shown in Fig. 7.4. Matrix-assisted

laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), NeoSickle[®], also has potential for neonatal screening [33].

Cellulose acetate electrophoresis can also be used but the eluate of a dried blood spot may be too dilute for this technique and, in addition, it is less sensitive than either HPLC or IEF for the detection of the low percentages of haemoglobin A that may be present in premature neonates. It is also less sensitive for the detection of small amounts of variant haemoglobins, levels down to 4% being detected in one study whereas HPLC and IEF detected haemoglobin variants down to levels of less than 2% [30]. Haemoglobin Bart's is unstable on storage and may therefore be missed, regardless of technique, if there is delay in testing [25]. In selecting a method for neonatal screening, consideration must be given to the workload and to whether it is convenient to use the same technique both for neonatal screening and for other screening. A consideration of both the requirement for sensitivity and the need to use the same instrumentation for other purposes means that HPLC is often the technique chosen. When mass spectrometry is already in use for screening for metabolic disorders, it may be chosen. In the UK, the selected methods detect haemoglobins S, C, D-Punjab, O-Arab and E; probable β thalassaemia major is also reported and further investigated [32]. The use of sensitive techniques is particularly important in screening premature babies in whom the percentage of haemoglobin A or of any variant haemoglobin is likely to be low. Screening of these babies should be done soon after birth to avoid the possibility of a sample being taken after a blood transfusion. The presence of more haemoglobin A than F or a prominent haemoglobin A₂ band in a neonatal sample should raise the possibility that there has been contamination with maternal blood or that a post-transfusion sample has been sent to the laboratory.

All haemoglobin variants detected by the initial screening method should be further investigated by a supplementary alternative method to make their presumptive identification more reliable. It should be noted that a sickle solubility test should not be used in neonates because

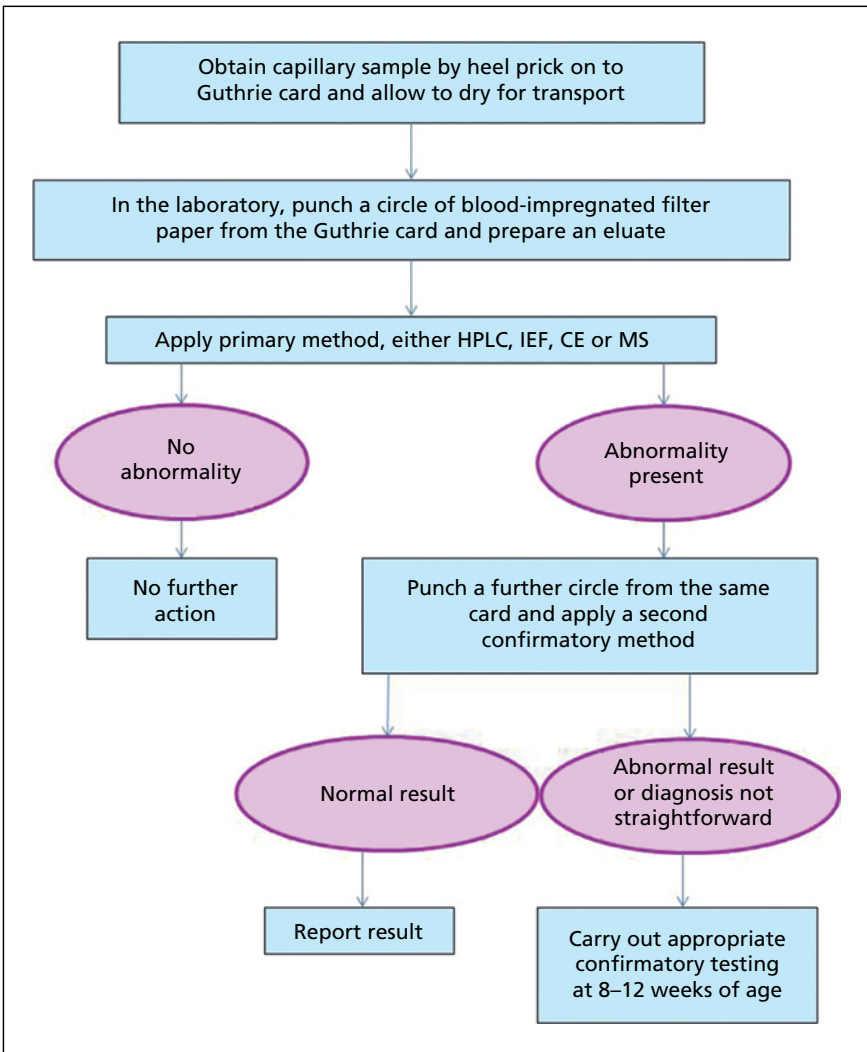


Fig. 7.4 A protocol for neonatal screening. IEF, isoelectric focusing; MS, mass spectrometry. If the baby has been transfused *in utero* or before a blood sample has been taken, follow an alternative policy of DNA analysis for the β^S gene.

of the high probability of false negative results. Potentially significant haemoglobinopathies should be confirmed by a second sample, conveniently around the age of 6–7 weeks (testing to be completed by eight weeks so there is no delay in commencing prophylactic penicillin). Repeat testing should also be performed on all babies whose initial sample showed no haemoglobin A. In addition, it is prudent to repeat tests if the predominant haemoglobin present is haemoglobin F with very small amounts of

haemoglobins A and S, since it can be difficult to distinguish sickle cell trait from sickle cell/ β^+ thalassaemia in this circumstance. The initial report on such a sample should be circumspect. The detection of only haemoglobins S and F in a neonate is most often attributable to sickle cell anaemia. However, compound heterozygosity for haemoglobin S and either β^0 thalassaemia or deletional HPFH also produces this pattern. In addition, it has been noted that some babies with sickle cell/ β^+ thalassaemia compound

heterozygosity also have only S and F detectable at birth, particularly if cellulose acetate electrophoresis is the detection method employed [34]. Studies on parental samples can help distinguish S/HPFH from clinically significant conditions and avoid unnecessary follow-up and further testing of these babies [35].

An essential part of any neonatal screening programme is a well organised system of follow-up and appropriate management of babies found to have a significant abnormality. Information and an appropriate explanation must also be given to the parents of babies found to have sickle cell trait or other heterozygous conditions. It is important for all those involved in neonatal screening schemes to remember that β thalassaemia trait will not be detected by testing in neonates or young infants. If it is known that one or both parents has β thalassaemia trait then this should be borne in mind when issuing a report.

Problems and pitfalls in neonatal diagnosis

Problems and pitfalls in neonatal diagnosis include: (i) baby not tested because of arrival in a country as a neonate or in early infancy; (ii) diagnosis missed when testing is selective rather than universal; (iii) intrauterine or neonatal transfusion prior to test; (iv) follow-up testing not done; and (v) parents not informed of carrier state.

Although it is desirable to inform parents when a carrier state is detected, not all European countries have always permitted this [27].

Preanaesthetic testing

It is important to detect all patients with sickle cell disease before anaesthesia in order to ensure that the necessity for preoperative blood transfusion is considered and that the patient is kept warm, well hydrated and well oxygenated both during surgery and in the post-operative period. Although patients with sickle cell anaemia and other severe sickling disorders will usually already have been diagnosed prior to presenting with a condition requiring immediate surgery

and anaesthesia, this is not necessarily so of patients with sickle cell/haemoglobin C disease and sickle cell/ β^+ thalassaemia. Such patients may have a normal Hb so that diagnosis, in an emergency situation, cannot rest on a sickle solubility test and full blood count (FBC) alone. Supplementing these tests with a blood film makes provisional identification of these compound heterozygous states more accurate.

It is also conventional to test any patient at risk of having sickle cell trait prior to anaesthesia, in order to ensure that hypoxia does not occur during surgery. It could be argued that *no* patient should be permitted to become hypoxic and that sickle screening is therefore unnecessary if sickle cell disease can be excluded. However, the detection of sickle cell trait can also be relevant to the prolonged application of a tourniquet and if cell salvage techniques are planned, so prudent practice is to test all patients of an appropriate ethnic group.

In testing for haemoglobin S it is necessary to bear in mind the very wide range of ethnic groups in which this variant haemoglobin can occur (see Chapter 4). If routine surgery is being planned, all patients of an appropriate ethnic group should have an FBC and HPLC, capillary electrophoresis or cellulose acetate electrophoresis, supplemented when a relevant abnormality is detected by a sickle solubility test. If emergency anaesthesia is required, the patient should be assessed for clinical features suggestive of an undiagnosed sickling disorder. If there are no such features, an FBC and sickle solubility test should be performed; if the Hb is reduced, a blood film should be examined. If the patient has clinical features compatible with a sickling disorder, FBC, blood film and sickle solubility test should be performed. The purpose of the blood film in these circumstances is to facilitate the diagnosis of patients with sickle cell disease with a normal Hb who might otherwise be assumed to have sickle cell trait.

When resources and time permit, a definitive diagnosis of sickle cell disease should be made prior to surgery. This is more likely to be feasible in laboratories using HPLC or capillary electrophoresis as the primary diagnostic method rather than cellulose acetate electrophoresis. If a

definitive diagnosis cannot be made rapidly, but sickle cell disease is suspected, surgery should proceed on the assumption that the patient does have sickle cell disease and appropriate attention should be paid to oxygenation and hydration. When full testing is not possible in an emergency situation, it is important to ensure that an adequate pretransfusion sample

is available for full testing on the next working day. A laboratory protocol for preanaesthetic testing is shown in Fig. 7.5.

These procedures will mean that the provisional diagnosis is correct in the majority of patients. Some patients with sickle cell disease with a high percentage of haemoglobin F or sickle cell/ β^+ thalassaemia with a high

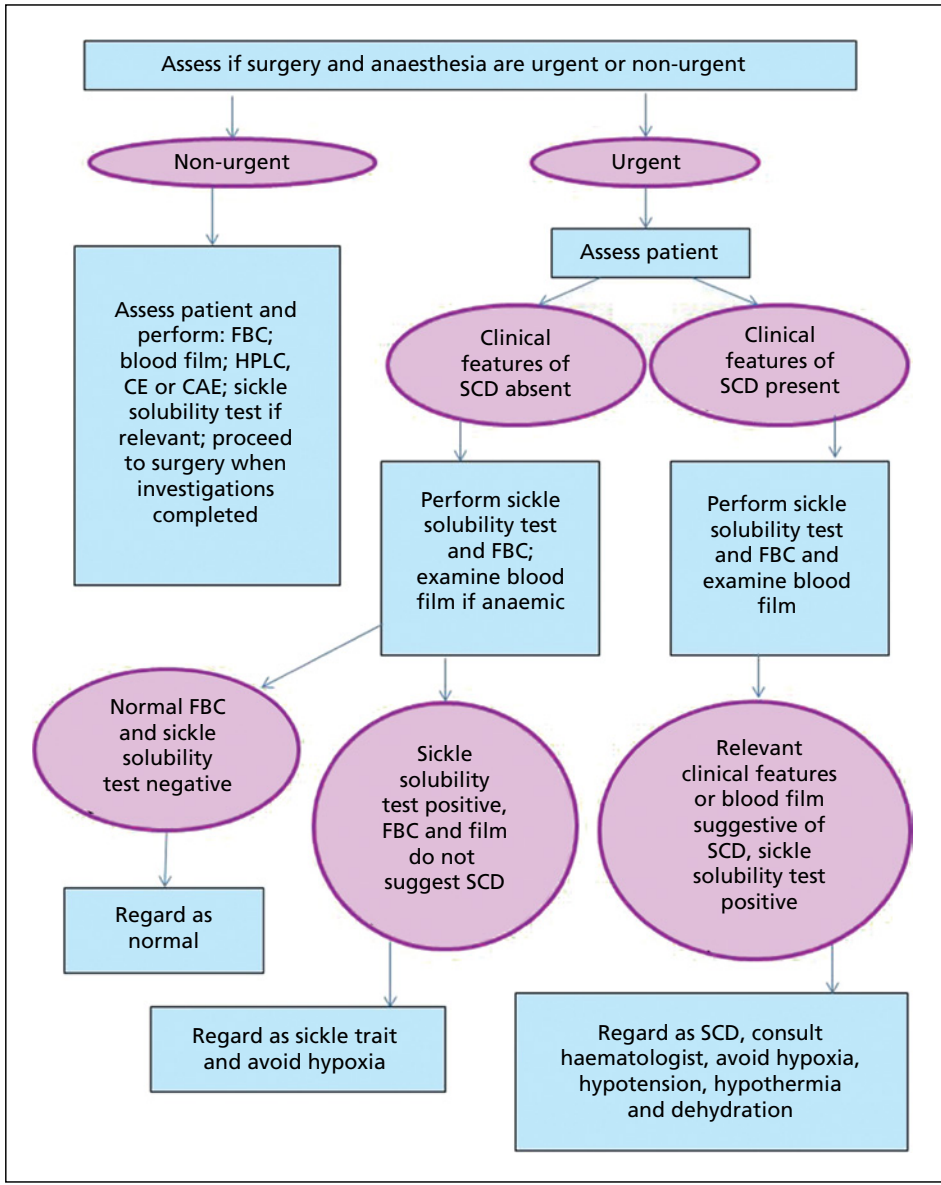


Fig. 7.5 A protocol for preanaesthetic testing to detect patients with sickle cell trait or sickle cell disease prior to routine or emergency surgery. CAE, cellulose acetate electrophoresis; SCD, sickle cell disease.

percentage of haemoglobin A may be missed but they are the patients most likely to have mild disease and least likely to suffer complications in relation to surgery and anaesthesia. It should also be noted that false negative results with a sickle solubility test can be seen in young infants. However, a false negative test is only expected with quite a low haemoglobin S percentage when anaesthetic complications would be less likely. Definitive testing by HPLC, capillary electrophoresis or cellulose acetate electrophoresis is required but emergency surgery can proceed.

Problems and pitfalls in preanaesthetic testing

Problems and pitfalls in preanaesthetic testing include: (i) failure to test at a preassessment clinic before planned surgery; (ii) failure to test all patients of appropriate ethnic origin; and (iii) failure to recognise that a patient has sickle cell disease (e.g. sickle cell/haemoglobin C compound heterozygosity) rather than sickle trait.

Other haemoglobinopathy investigations

Investigation of haemolytic anaemia

Haemolytic anaemia can be consequent on the presence of an unstable haemoglobin. When this is suspected the following tests should be performed:

- FBC, blood film and reticulocyte count;
- HPLC, capillary electrophoresis or cellulose acetate electrophoresis;
- isopropanol and/or heat instability test;
- haemoglobin A₂ estimation;
- a test for Heinz bodies, repeated after incubation for 24 hours at 37°C, if initially negative;
- family studies;
- tests to exclude other causes of a haemolytic anaemia;
- DNA analysis.

Occasionally, when a variant haemoglobin is very unstable, instability tests are negative and diagnosis rests on DNA analysis.

Investigation of unexplained cyanosis

Unexplained cyanosis can be caused by a low oxygen affinity haemoglobin, methaemoglobinemia or sulphhaemoglobinemia. Some inherited methaemoglobins are also unstable. Relevant investigations include:

- FBC, blood film and reticulocyte count;
- HPLC, capillary electrophoresis or cellulose acetate electrophoresis;
- cellulose acetate electrophoresis after conversion of all haemoglobin to methaemoglobin;
- isopropanol and/or heat instability test;
- spectrophotometry for the detection and quantification of sulph- and methaemoglobin;
- oxygen dissociation curve and estimation of $P_{50}O_2$;
- partial pressure of oxygen in arterial blood to exclude hypoxia as a cause of cyanosis;
- family studies;
- DNA analysis.

Investigation of unexplained polycythaemia

Polycythaemia can be consequent on a high affinity haemoglobin or, rarely, on homozygosity for deletional HPFH. Some high oxygen affinity haemoglobins are also unstable. Relevant investigations include:

- FBC, blood film and reticulocyte count;
- HPLC, capillary electrophoresis or cellulose acetate electrophoresis;
- isopropanol and/or heat instability tests;
- oxygen dissociation curve and estimation of $P_{50}O_2$;
- family studies;
- DNA analysis;
- investigation to exclude other causes of polycythaemia.

Identification of an unknown variant haemoglobin

Uncommon haemoglobins that are not readily identifiable are occasionally detected in screening programmes. Variant haemoglobins may also be detected because of an aberrant result when measuring haemoglobin

A_{1c} for monitoring of diabetes. It is not always necessary to identify a variant haemoglobin but it is necessary to determine whether or not it is of potential clinical significance. Routine tests should be applied, as shown in Figs 7.1 and 7.2, in order to identify the common variant haemoglobins likely to be clinically important, including haemoglobins S, C, D-Punjab, E, O-Arab, Lepore and Constant Spring. If the variant haemoglobin remains unidentified after one of these protocols, the following factors should be considered:

- whether there is any personal or family history suggestive of a clinically significant variant haemoglobin (e.g. anaemia, jaundice, polycythaemia);
- whether there is an elevated reticulocyte count or any blood film abnormality suggestive of haemolysis;
- whether there are red cell indices suggestive of a thalassaemic condition;
- whether the patient is in the reproductive age range or has close relatives who might be considering pregnancy.

Further tests that might be indicated include a sickle solubility test (since not all sickling haemoglobins have the elution time or electrophoretic mobility of haemoglobin S) and a test for an unstable haemoglobin. Other tests that might be selectively applied if the variant haemoglobin has not been identified by HPLC, capillary electrophoresis or cellulose acetate electrophoresis include:

- oxygen dissociation curve and determination of $P_{50}O_2$;
- haemoglobin absorption spectrum;
- electron spray mass spectrometry;
- DNA sequencing or other molecular techniques.

With the increasing availability of mass spectrometry and DNA sequencing, there is no longer a significant role for globin chain electrophoresis, tryptic digestion and peptide fingerprinting or globin chain synthesis studies. Increasingly, DNA analysis is used as a first-line test if there is a need to positively identify haemoglobin variants which cannot be identified by HPLC or IEF alone.

Haemoglobinopathy investigations in a resource-poor setting

Appropriate investigations in a resource-poor setting will depend on the prevalence of various haemoglobinopathies and on financial and other constraints. In all such settings, an FBC, including MCV or MCH, is highly desirable. When an automated counter is not available, a modified osmotic fragility test (Naked Eye Single Tube Red cell Osmotic Fragility Test, NESTROFT) can be used to screen for α and β thalassaemia trait. Abnormal results are found also in iron deficiency and sickle cell trait. If an automated counter is available, use of this screening test may not be advised, since false negative results are obtained in a small percentage of cases of β thalassaemia heterozygosity. However, a study from Thailand found that the osmotic fragility test showed 95% sensitivity for the detection of α^0 or β thalassaemia while the MCV, with a cut-off point of 78.1 fl, showed 93% sensitivity [36]. Sensitivity for detection of β thalassaemia trait is reduced if there is coinheritance of β thalassaemia with both α thalassaemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency; sensitivity is likely to drop below 93% in some populations [37]. If there is inheritance of South-East Asian ovalocytosis in addition to α thalassaemia and G6PD deficiency, sensitivity in some populations could drop to less than 70% while coinheritance of β thalassaemia trait and South-East Asian ovalocytosis alone is associated with a sensitivity of 80–90% [37].

The dichlorophenolindophenol (DCIP) test can be used to screen for haemoglobin E heterozygosity, haemoglobin E homozygosity and haemoglobin E/ β thalassaemia compound heterozygosity; positive results are also obtained in haemoglobin H disease and with unstable haemoglobins. Modifications of the DCIP tests improve sensitivity and specificity [38]. In Thailand, initial antenatal screening for α^0 and β thalassaemia and haemoglobin E is by means of either a modified osmotic fragility test or red cell indices plus a DCIP test; testing for haemoglobin H inclusions and immunochromatographic detection of ζ chain or haemoglobin Bart's can have a supplementary role [39]. In a

Sri Lankan study, when screening for haemoglobin E heterozygosity, sensitivity increased from 89% to 98% when an MCV < 80 fl or MCH < 27 pg was supplemented by a red cell distribution width (RDW) > 14.45% [40]. Point-of-care tests applicable to some types of α^0 thalassaemia in a resource-poor setting have also been developed (see page 112).

In countries where sickle cell disease is the main clinical problem, a sickle solubility test and cellulose acetate electrophoresis at alkaline pH [41] or a point-of-care test (see later) may suffice for basic diagnosis. When β thalassaemia is a significant problem, HPLC or capillary electrophoresis is likely to be preferred to cellulose acetate electrophoresis since it permits measurement of haemoglobin A₂ as well as provisional identification of haemoglobin S and other variant haemoglobins. Newborn screening in a number of countries in sub-Saharan Africa is now being developed, in some cases facilitated by the Consortium for Newborn Screening in Africa (CONSA) [42].

Point-of-care testing for haemoglobin S

Point-of-care testing for detection of haemoglobins S and possibly also haemoglobin C can be useful in a resource-poor setting, sometimes including use in outreach clinics. An ideal test will be fast, inexpensive, not dependent on an electricity supply, refrigeration or expensive equipment, and with a high sensitivity. A high specificity is also desirable since this reduces the need for follow-up testing.

A novel point-of-care test for the detection of sickle cell disease depends on the ability of normal cells to move through a piece of modified chromatography paper while sickled cells cannot. A drop of blood is mixed with sickle solubility reagents and a drop of the mixture is then applied to the paper. Normal, sickle cell trait (AS) and sickle cell anaemia (SS) can be distinguished visually, with sickle cell/haemoglobin C disease (SC) results falling between AS and SS [43]. A modification of the method makes it suitable for neonatal screening, a study in Angola finding 100% sensitivity and 83% specificity for detection of sickle cell anaemia [44].

Another novel method uses density separation of red cells, with the presence of dense cells permitting sickle cell anaemia and sickle cell/haemoglobin C disease to be distinguished from each other and from sickle cell trait or normal with a sensitivity of 90–91% and a specificity of 88–91%; sickle cell trait and normal samples are not distinguished from each other [45].

A rapid point-of-care immunoassay, Sickie SCAN[®], a lateral flow test using polyclonal antibodies, distinguishes between haemoglobins S, C and 'haemoglobin other than S or C' (usually haemoglobin A) [46]. It is applicable to dried blood spots and may thus be suitable not only for screening for carriers but also for testing of neonates in resource-poor settings. No power source is needed. A preliminary study in Nigeria showed 100% sensitivity in detection of sickle cell disease (one SS, one SC) from the age of nine months upwards (younger infants not tested); SS, SC and AS were generally distinguished [47]. A subsequent Nigerian study found 95% sensitivity and 99% specificity [48, 49].

Another rapid point-of-care test, HemoTypeSC[™], uses monoclonal antibodies and a competitive lateral flow immunochromatographic assay to recognise the presence of haemoglobins S, C and 'not S or C' (mainly haemoglobin A) [48–51]. It requires only 15 μ l of blood and will distinguish between haemoglobin A plus S (either sickle cell trait or haemoglobin S/ β^+ thalassaemia); haemoglobin A plus C (either haemoglobin C trait or haemoglobin C/ β^+ thalassaemia); haemoglobin S plus C; haemoglobin S only (sickle cell anaemia or haemoglobin S/ β^0 thalassaemia); haemoglobin C only (either haemoglobin C homozygosity or haemoglobin C/ β^0 thalassaemia); and 'haemoglobin other than S or C only' (mainly haemoglobin A only) [50]. Testing is reliable irrespective of the presence of haemoglobin F, even at high levels [50]. It requires no instrumentation and no power source so is useful in resource-poor settings where the only common variant haemoglobins are S and C. In a large study carried out mainly in Ghana, the sensitivity and specificity for SS/S β^0 thalassaemia

and CC were both 100%, while for SC the sensitivity was 97% and the specificity 100% [51]. For A+S, A+C and A only the sensitivity and specificity were both above 99% [51]. A Ugandan study similarly found a very high degree of accuracy in the detection of hemoglobins A and S in patients from the age of one month upwards [52]. A Nigerian study found 94% sensitivity and 99% specificity [48]. In contrast to Sickie SCAN, interpretation is counterintuitive since the presence of a line indicates the *absence* of the relevant haemoglobin [49]. Both Sickie SCAN and HemoTypeSC have been found effective in real-life neonatal screening in Angola with accuracy of 98.3% and 95.3% respectively when compared with a reference method [53].

Gazelle™ Hb Variant is a miniaturised microchip-based, cellulose acetate electrophoresis point-of-care test [49]. In contrast to the lateral flow tests above, it requires a battery as a power source. It can detect haemoglobins A, F, S and C/E/A₂.

Microfluidic electrophoretic techniques such as HemeChip are being developed. HemeChip, which is battery powered, is suitable as a point-of-care test, is rapid and separates haemoglobins A, F, S and C/E/A₂ [54].

Avoiding errors in haemoglobinopathy diagnosis and screening

Errors in haemoglobinopathy diagnosis and screening can have profound effects on patients and their families, leading, for example, to continuing pregnancy when a fetus has haemoglobin Bart's hydrops fetalis or to the birth of a baby with β thalassaemia major. It is important that guidelines and Standard Operating Procedures are followed strictly. It must also be recognised that screening is not the same as making a definitive diagnosis, and screening programmes, almost by definition, will miss some haemoglobinopathy cases with unusual or atypical features. Some sources of error are shown in Table 7.5 and detailed examples are given in reference [55].

Table 7.5 Sources of error in haemoglobinopathy diagnosis and screening.

Blood sample from wrong patient
Identity fraud
Conception from donor egg or donor sperm not revealed
Test not done when indicated, e.g. failure to detect that a pregnant woman is heterozygous for both β thalassaemia and α^0 thalassaemia or failure to test the partner of a pregnant woman
Recent transfusion not known to laboratory
Previous bone marrow transplantation
Contamination with blood from a different patient
Transcription error in laboratory
Haemoglobin A ₂ falsely low, e.g. in the presence of δ thalassaemia or when haemoglobin A ₂ and haemoglobin A ₂ variant are not summed in a patient with an α chain variant
Laboratory results misinterpreted in laboratory
Clear summary of significance of results not given to clinician.

Internet resources

EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies: www.ncbi.nlm.nih.gov/pmc/articles/PMC4666573

Sickle cell and thalassaemia screening: handbook for antenatal laboratories: www.gov.uk/government/publications/sct-screening-handbook-for-antenatal-laboratories

Sickle cell and thalassaemia screening: handbook for newborn laboratories: www.gov.uk/government/publications/sct-screening-handbook-for-newborn-laboratories

Population screening programmes: NHS sickle cell and thalassaemia (SCT) screening programme: www.gov.uk/government/collections/nhs-sickle-cell-and-thalassaemia-sct-screening-programme

Sickle in Africa: www.sickleinafrica.org

Thalassaemia International Federation: <https://thalassaemia.org.cy>

Consortium for Newborn Screening in Africa: www.hematology.org/global-initiatives/consortium-on-newborn-screening-in-africa

Check your knowledge

One to five answers may be correct. Answers to most questions can be either found in this chapter or deduced from information given. Answers are given on page 422.

- 7.1 An antenatal haemoglobinopathy screening programme should detect
 - (a) α^0 thalassaemia
 - (b) α^+ thalassaemia
 - (c) β^0 thalassaemia
 - (d) β^+ thalassaemia
 - (e) $\delta\beta$ thalassaemia
- 7.2 Sickle cell disease, which could complicate surgery and anaesthesia, could be observed in
 - (a) Arabs
 - (b) South Asians
 - (c) southern Europeans
 - (d) North Africans
 - (e) African Caribbeans
- 7.3 Haemoglobin electrophoresis plus HPLC at six weeks of age could provide a definitive diagnosis of
 - (a) β thalassaemia trait
 - (b) sickle cell/hereditary persistence of fetal haemoglobin
 - (c) sickle cell/ β^0 thalassaemia
 - (d) sickle cell/haemoglobin C disease
 - (e) sickle cell trait
- 7.4 It is important that an antenatal haemoglobinopathy screening programme detects
 - (a) haemoglobin C trait
 - (b) haemoglobin D-Punjab trait
 - (c) haemoglobin E trait
 - (d) haemoglobin G-Philadelphia trait
 - (e) haemoglobin Lepore trait
- 7.5 In an antenatal haemoglobinopathy screening programme, investigation for α^0 thalassaemia trait should be carried out in
 - (a) Chinese
 - (b) Laotians
 - (c) Cambodians
 - (d) Japanese
 - (e) Vietnamese
- 7.6 A patient with a high oxygen affinity haemoglobin could have normal results for
 - (a) cellulose acetate electrophoresis at alkaline pH
 - (b) oxygen dissociation curve
 - (c) agarose gel electrophoresis at acid pH
 - (d) measurement of $P_{50}O_2$
 - (e) isopropanol test
- 7.7 A neonatal haemoglobinopathy screening programme should aim to detect cases of
 - (a) α thalassaemia trait
 - (b) β thalassaemia trait
 - (c) sickle cell anaemia
 - (d) sickle cell/ β^0 thalassaemia compound heterozygosity
 - (e) hereditary persistence of fetal haemoglobin
- 7.8 In an antenatal haemoglobinopathy screening programme, DNA testing for α^0 thalassaemia trait should be carried out in
 - (a) Greeks
 - (b) Cypriots
 - (c) Spaniards
 - (d) Portuguese
 - (e) Austrians
- 7.9 Suitable primary methods for neonatal haemoglobinopathy screening include
 - (a) cellulose acetate electrophoresis at alkaline pH
 - (b) citrate agar or agarose gel electrophoresis at acid pH
 - (c) isoelectric focusing
 - (d) high performance liquid chromatography
 - (e) sickle solubility test
- 7.10 Emergency preanaesthetic screening should detect patients with
 - (a) sickle cell trait
 - (b) β thalassaemia trait
 - (c) sickle cell anaemia
 - (d) sickle cell/haemoglobin C disease
 - (e) haemoglobin C disease

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Answers to questions

- | | | | | |
|-----------|-----------|-----------|-----------|------------|
| 7.1 (a) T | 7.3 (a) F | 7.5 (a) T | 7.7 (a) F | 7.9 (a) T |
| (b) F | (b) F | (b) T | (b) F | (b) F |
| (c) T | (c) F | (c) T | (c) T | (c) T |
| (d) T | (d) T | (d) F | (d) T | (d) T |
| (e) T | (e) T | (e) T | (e) F | (e) F |
| 7.2 (a) T | 7.4 (a) T | 7.6 (a) T | 7.8 (a) T | 7.10 (a) T |
| (b) T | (b) T | (b) F | (b) T | (b) F |
| (c) T | (c) T | (c) T | (c) F | (c) T |
| (d) T | (d) F | (d) F | (d) F | (d) T |
| (e) T | (e) T | (e) T | (e) F | (e) F |

8 Self-assessment – test cases

All the case studies are based on real patients presenting real diagnostic problems. The reader is advised that not all are straightforward. Careful thought and, if necessary, reference back to earlier chapters are advised before looking at the answers given in the second half of this chapter.

Exercise 8.1

You are provided with a diagrammatic representation of results of haemoglobin electrophoresis on cellulose acetate at alkaline pH and haemoglobin electrophoresis on agarose gel at acid pH and with the results of a sickle solubility test on a control sample and samples from patients 1–11.

Give the most likely explanation or explanations for each case. No patient had been transfused and all were adults. (For patient 3, note the quantity of the variant haemoglobin.)

- Patient 1
- Patient 2
- Patient 3
- Patient 4
- Patient 5
- Patient 6
- Patient 7
- Patient 8
- Patient 9
- Patient 10
- Patient 11

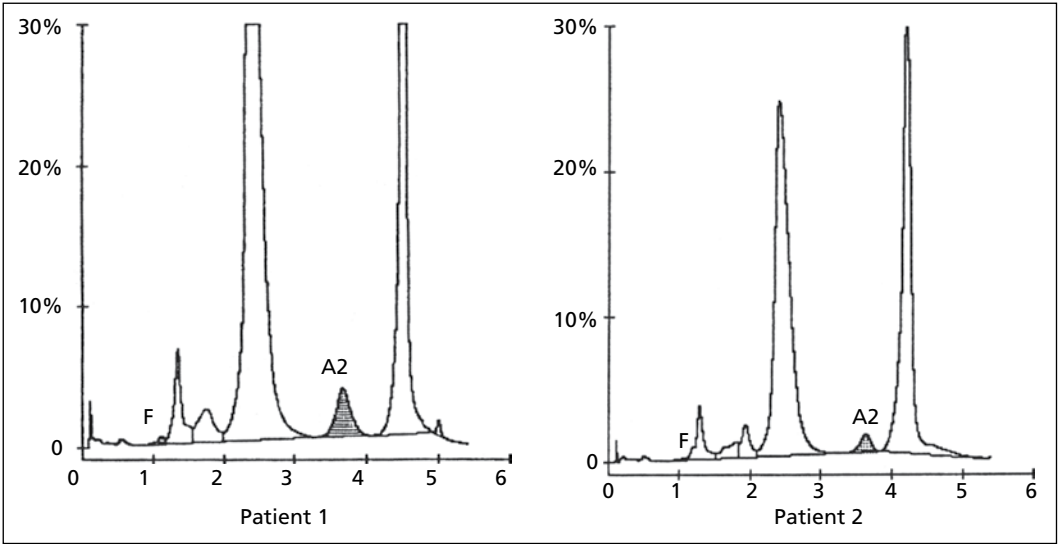
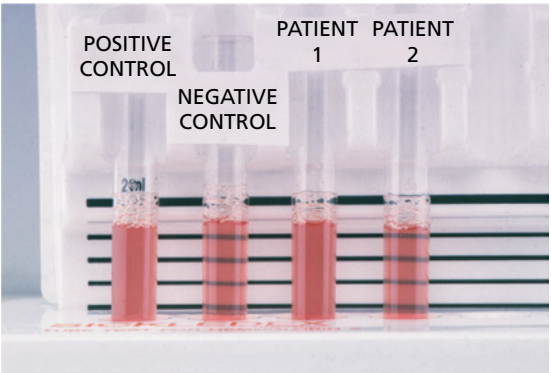
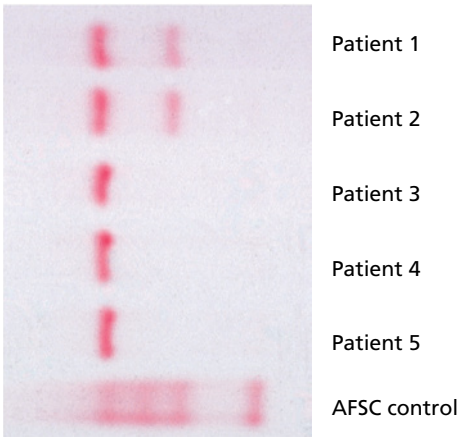
	Cellulose acetate electrophoresis – alkaline			Agarose gel electrophoresis – acid			Sickle solubility test
	A	F	S	F	A	S	
Control							
①							Positive
②							Negative
③							Negative
④							Positive
⑤							Positive
⑥							Positive
⑦							Negative
⑧							Negative
⑨							Negative
⑩							Positive
⑪							Negative

Exercise 8.2

You are provided with haemoglobin electrophoresis at alkaline pH, a sickle solubility test and a high performance liquid chromatography

(HPLC) chromatogram (Bio-Rad Variant) on two African Caribbean patients, patient 1 and patient 2. Give the most likely diagnosis in each.

Patient 1
Patient 2



Exercise 8.3

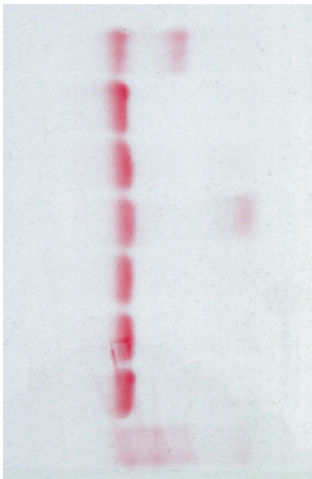
You are provided with a blood film and the results of cellulose acetate electrophoresis at alkaline pH (lane d) on a 33-year-old pregnant Bangladeshi woman with the following red cell indices: red cell count (RBC) $4.39 \times 10^{12}/l$, haemoglobin concentration (Hb) 110g/l, haematocrit (Hct) 0.32, mean cell volume (MCV) 74fl, mean

cell haemoglobin (MCH) 25.1pg and mean cell haemoglobin concentration (MCHC) 332g/l.

What is the most likely diagnosis?

What test would you perform to confirm the diagnosis?

What abnormality in the partner would be most likely to lead to serious disease in the fetus?



A S C/E

Exercise 8.4

A young woman was referred to a haematologist because of borderline anaemia (Hb between 100 and 115 g/l) and microcytosis that had not responded to oral iron. The patient stated that as far as she knew, her ancestry was totally English although her father had once been told that he had 'Spanish blood'. The haematologist stopped the iron therapy and a week later performed various tests with the following results.

RBC $5.37 \times 10^{12}/l$, Hb 116 g/l, MCV 69 fl, MCH 21.7 pg, MCHC 312 g/l, red cell distribution width (RDW) 19.2% (normal range

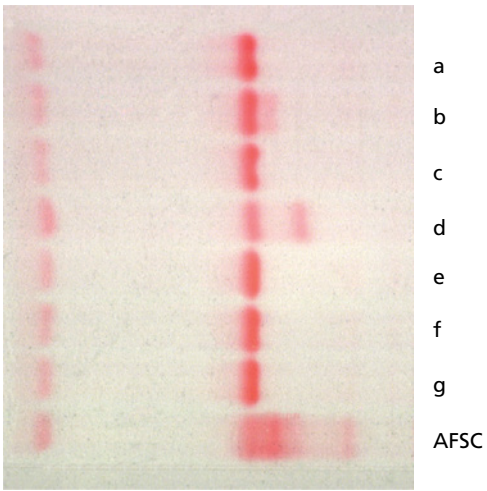
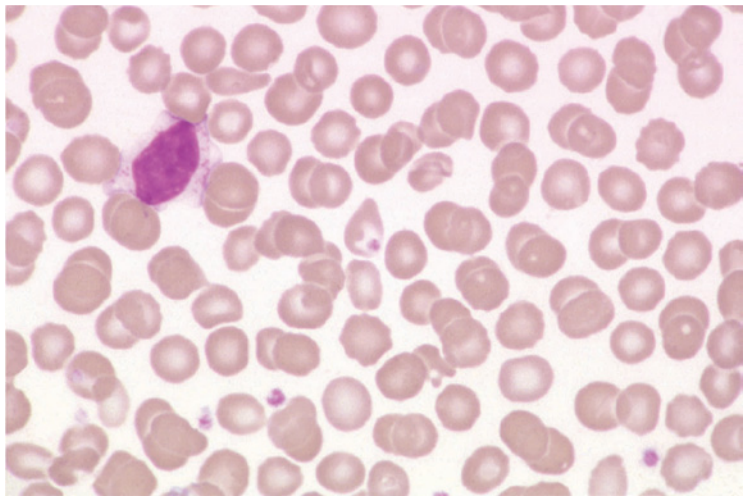
11.1–14.9) and serum ferritin 28 $\mu\text{mol}/l$ (normal range 10–300).

You are provided with a photograph of the blood film and the electrophoretic pattern on cellulose acetate at alkaline pH (lane b). The haemoglobin A₂ was estimated at 1.8% and 2.5% on two occasions (normal range 2.3–3.5%). Haemoglobin F was 11.4% by alkali denaturation with a heterogeneous distribution on a Kleihauer test.

What is the most likely diagnosis?

What advice should be given to the patient?

.....

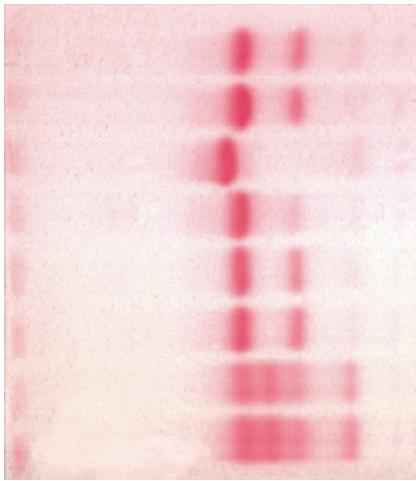
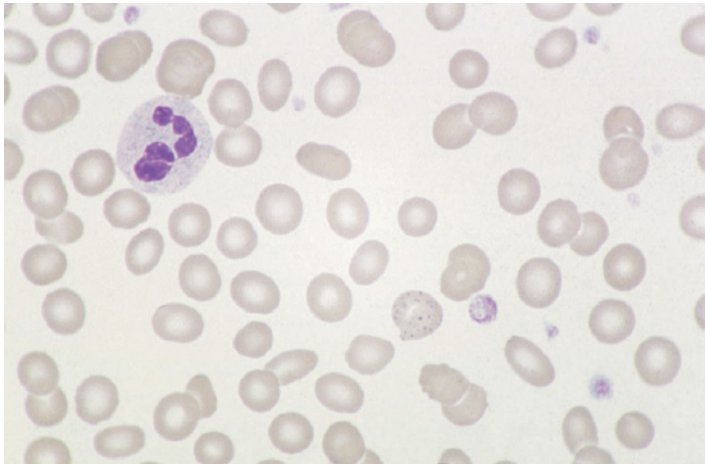


Exercise 8.5

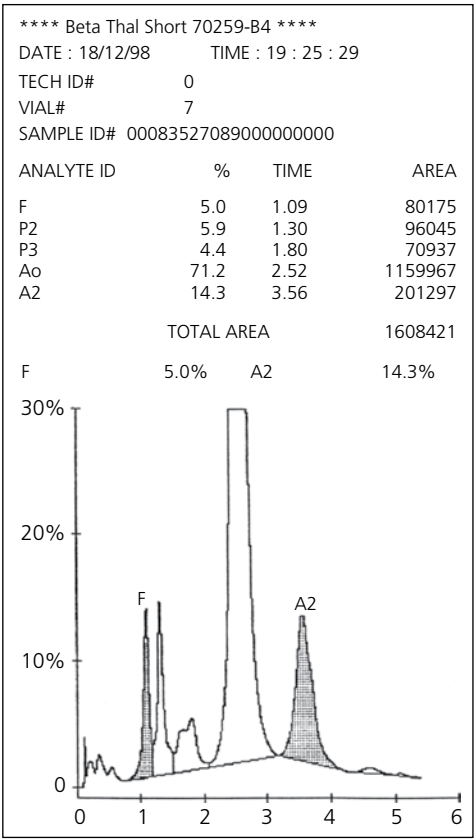
You are provided with the blood film, the electrophoretic pattern on cellulose acetate at alkaline pH (lane d) and the HPLC chromatogram (Bio-Rad Variant) of a 34-year-old woman from Gibraltar with the following red cell indices:

RBC $4.83 \times 10^{12}/l$, Hb 105 g/l, Hct 0.35, MCV 72 fl, MCH 21.7 pg and MCHC 301 g/l. Electrophoresis at acid pH was normal.

What is the most likely diagnosis?
What is the clinical significance of this result?
.....



a
b
c
d
e
f
AFSC
AFSC

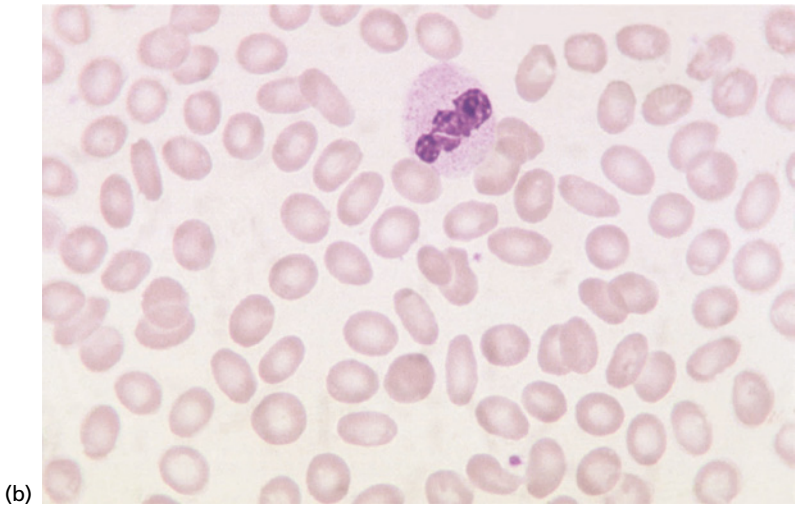
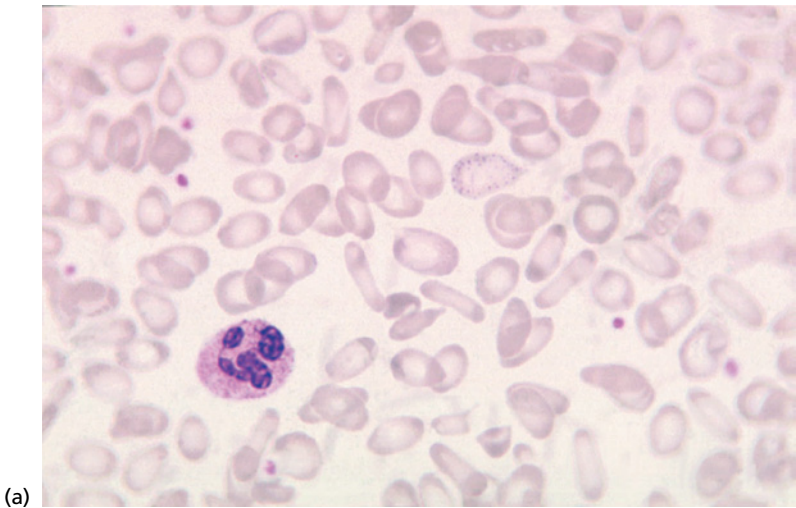


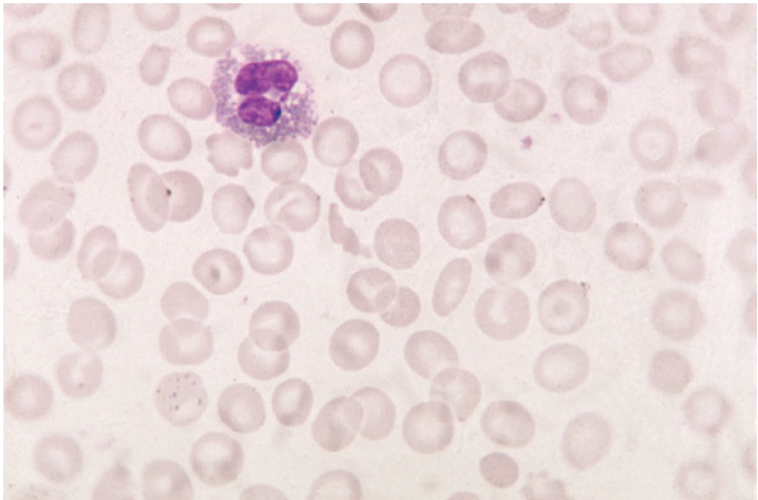
Exercise 8.6

You are provided with blood films, red cell indices and the results of haemoglobin electrophoresis on a middle-aged man, who was complaining of fatigue, and from three of his children.

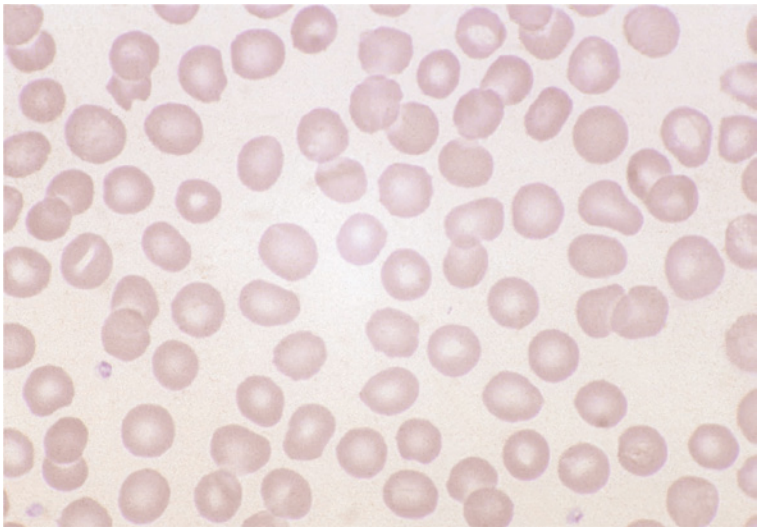
What is the most likely diagnosis in each family member?
Father
17-year-old daughter
15-year-old daughter
10-year-old son
(With thanks to Dr Francis Toolis)

Family member	RBC ($\times 10^{12}/l$)	Hb (g/l)	MCV (fl)	MCHC (g/l)	Haemoglobin electrophoresis
Father (a)	5.47	107	65	302	A + A ₂ (A ₂ 5.2%)
Daughter, aged 17 (b)	3.96	117	87	343	A + A ₂ (A ₂ 2.5%)
Daughter, aged 15 (c)	5.43	109	64	314	A + A ₂ (A ₂ 5.1%)
Son, aged 10 (d)	4.61	129	88	339	A + A ₂ (A ₂ 2.3%)





(c)



(d)

Exercise 8.7

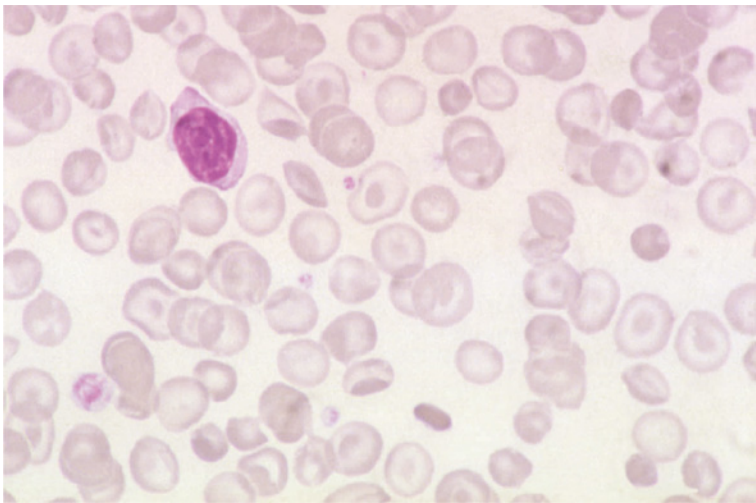
A 74-year-old white American woman was hospitalised because of back pain caused by osteoporosis. A blood film showed hypochromia and a full blood count (FBC) showed: RBC $2.9 \times 10^{12}/\text{l}$, Hb 69 g/l, Hct 0.21, MCV 74 fl, MCH 23 pg and RDW 18.4% (normal range 11.1–14.9). She was transfused two units of red cells and was discharged. Several weeks later she was readmitted with an Hb of 59 g/l and was found to have a bleeding gastric ulcer. She was transfused five units of blood over three days. Subsequently haemoglobin electrophoresis on cellulose acetate at alkaline pH showed a band with the mobility of haemoglobin S and a sickle solubility test was positive. Haemoglobins were quantified by HPLC as follows: haemoglobin A 89.7%, haemoglobin A₂ 2.9%, haemoglobin F 0.4% and haemoglobin S 7.0%.

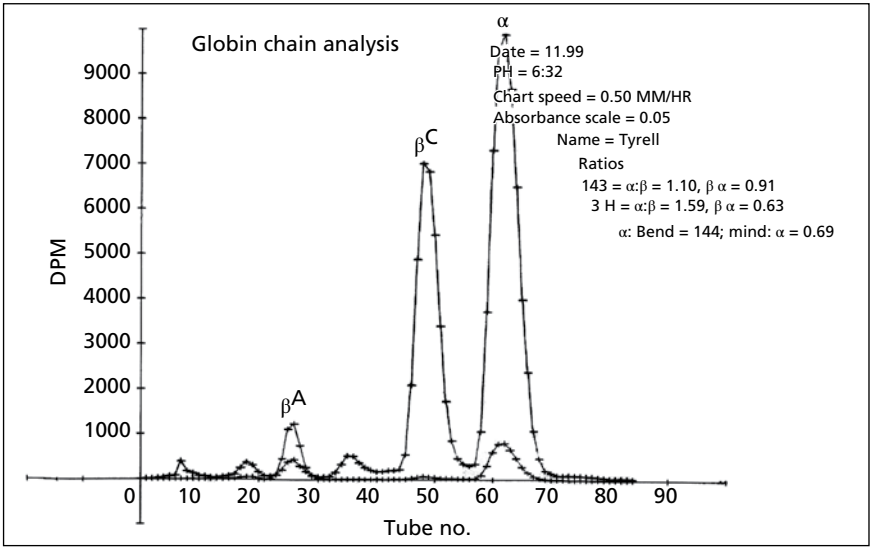
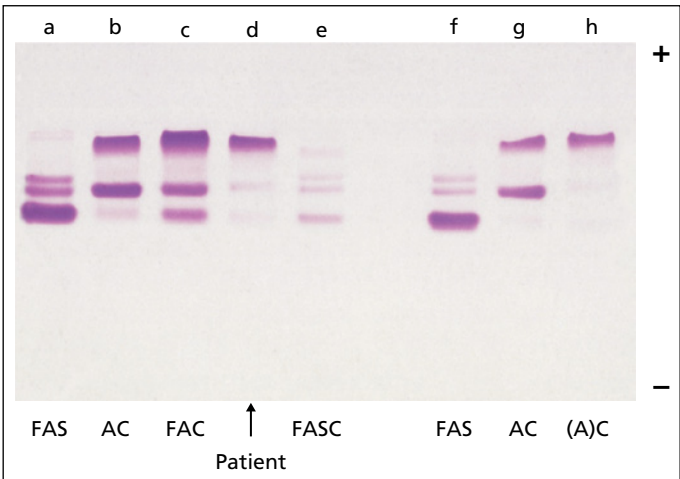
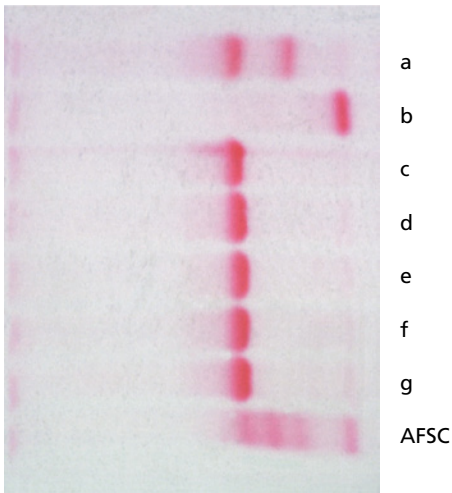
What explanations of these results would you consider and what further investigations would you perform?

Exercise 8.8

You are provided with photographs of a blood film, haemoglobin electrophoresis on cellulose acetate at alkaline pH (lane b), haemoglobin electrophoresis on agarose gel at acid pH (fourth lane from left) and the results of globin chain synthesis studies (with thanks to Professor Lucio Luzzatto) on an African Caribbean patient with the following red cell indices: RBC $5.43 \times 10^{12}/\text{l}$, Hb 107 g/l, Hct 0.34, MCV 63 fl, MCH 19.7 pg and MCHC 312 g/l.

What is the diagnosis?





Exercise 8.9

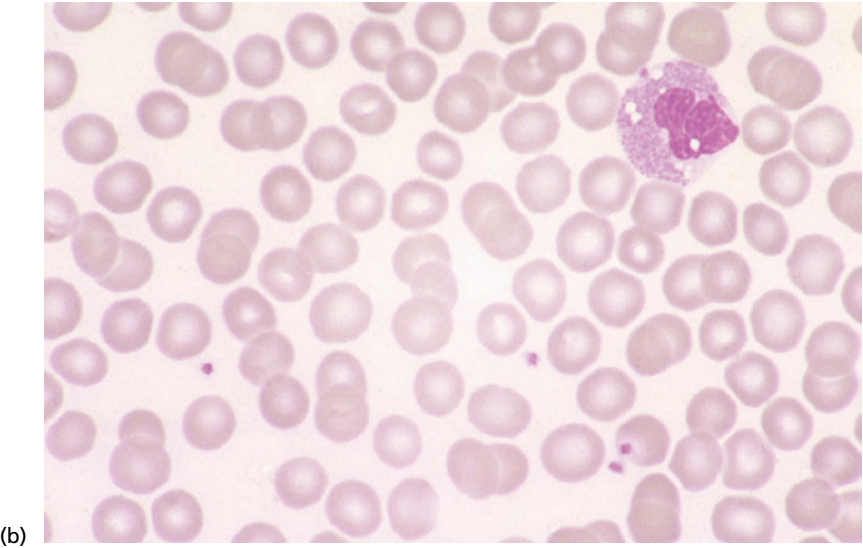
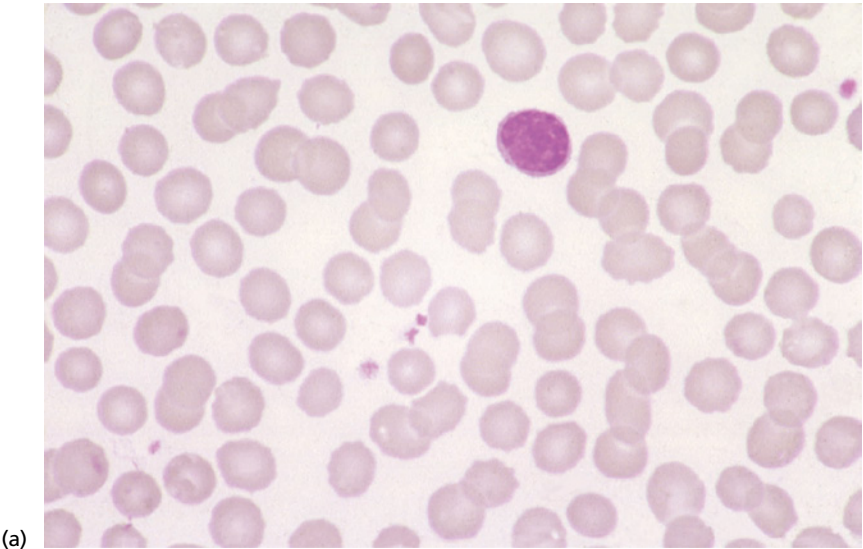
You are provided with red cell indices and photographs of blood films, cellulose acetate electrophoresis at alkaline pH and results of sickle solubility tests on a child and his parents.

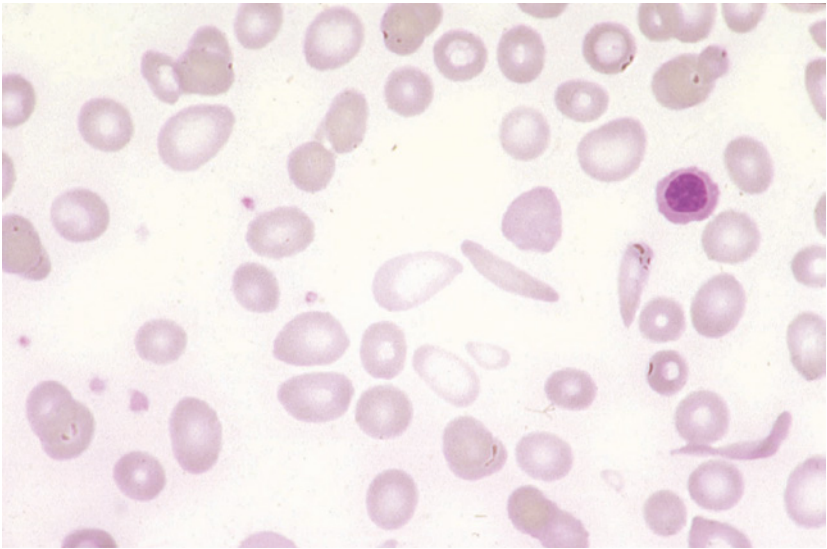
In the cellulose acetate electrophoresis the mother is lane a, the father lane b and the child lane f.

What is the likely diagnosis in each family member?

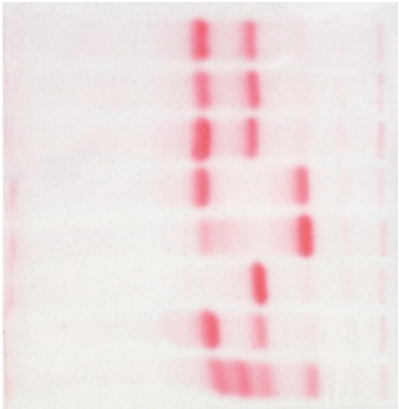
Mother
Father
Child

	RBC ($\times 10^{12}/l$)	Hb (g/l)	MCV (fl)	MCH (pg)	Sickle solubility test
Mother (a)	4.39	117	80	26.6	Negative
Father (b)	4.96	146	88	29.4	Positive
Child (c)	2.05	72	98	35.2	Positive





(c)



a
b
c
d
e
f
g
AFSC

Exercise 8.11

A young man had a routine blood count performed. This was noted to show abnormal red cell indices, leading to haemoglobin electrophoresis being performed. Three of the patient's grandparents were of Indian ethnic origin. The other was half-Indian and half-Chinese. There was no history of consanguinity. Red cell indices were: RBC $5.56 \times 10^{12}/l$, Hb 110 g/l, Hct 0.36, MCV 65 fl, MCH 19.8 pg and MCHC 307 g/l. The haemoglobin A₂ was 5.5%. You are provided

with a photograph of the patient's blood film and of haemoglobin electrophoresis on cellulose acetate at alkaline pH (lane c).

Suggest two possible explanations of the abnormality observed.

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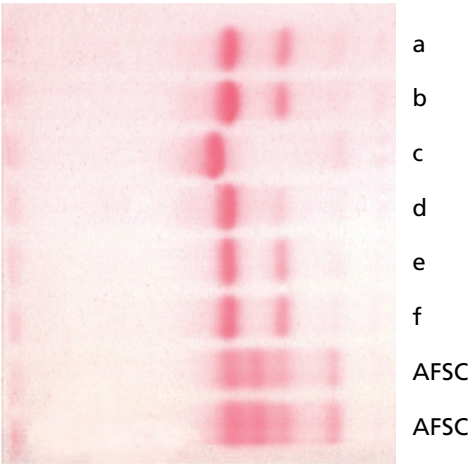
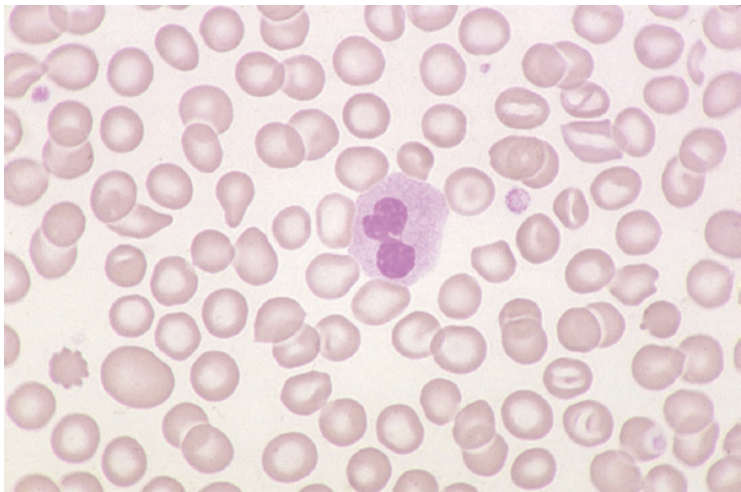
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What is the likely clinical significance?

.....

.....

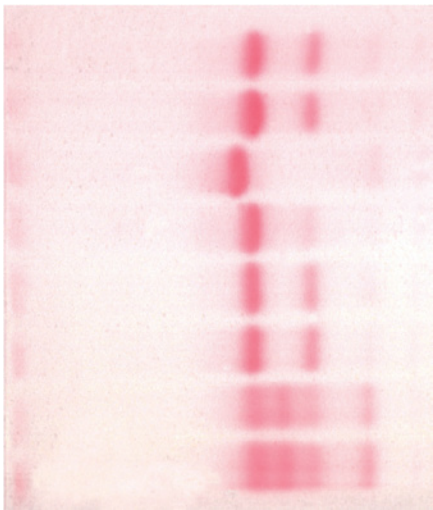
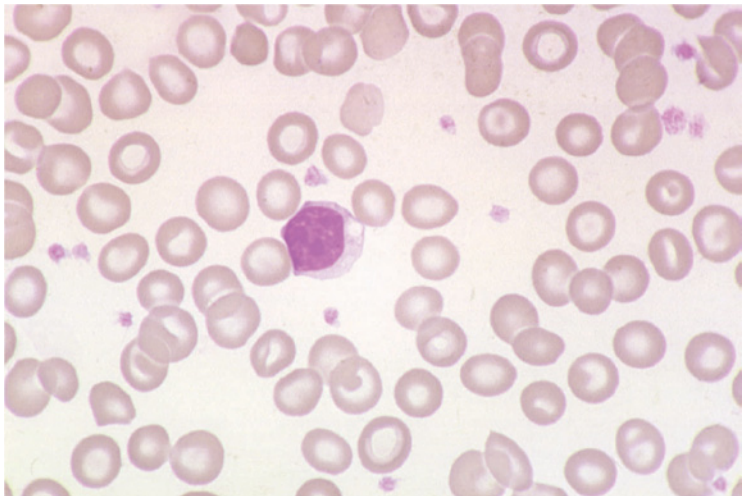


Exercise 8.12

A 23-year-old African Caribbean woman had a positive sickle solubility test and the following red indices: RBC $4.42 \times 10^{12}/l$, Hb 115g/l, Hct 0.35, MCV 78 fl, MCH 26pg and MCHC 318g/l. You are provided with the photographs of a

blood film and a cellulose acetate electrophoretic strip at alkaline pH (lane e).

What is the most likely explanation of the abnormal red cell indices and electrophoretic abnormality?



a
b
c
d
e
f
AFSC
AFSC

Exercise 8.13

A 67-year-old man of northern European origin presented with a history of breathlessness and ankle oedema. On physical examination, he was pale but no other abnormality was detected. His FBC showed white cell count (WBC) $5.7 \times 10^9/l$, RBC $5.3 \times 10^{12}/l$, Hb 94 g/l, Hct 0.36, MCV 68.6 fl, MCH 17.7 pg, MCHC 261 g/l and platelet count $651 \times 10^9/l$. You are provided with photographs of his peripheral blood film and his bone marrow aspirate.

The bone marrow was hypercellular with increased numbers of megakaryocytes, some of which were hypolobated. The bone marrow differential count showed 91% erythroid cells; 40%

of remaining cells were blast cells. Erythroblasts showed increased siderotic granulation including a low percentage of ring sideroblasts. Haemoglobin electrophoresis showed 15% haemoglobin H and a haemoglobin H preparation showed typical inclusions. The $\alpha:\beta$ chain synthesis ratio was 0.49.

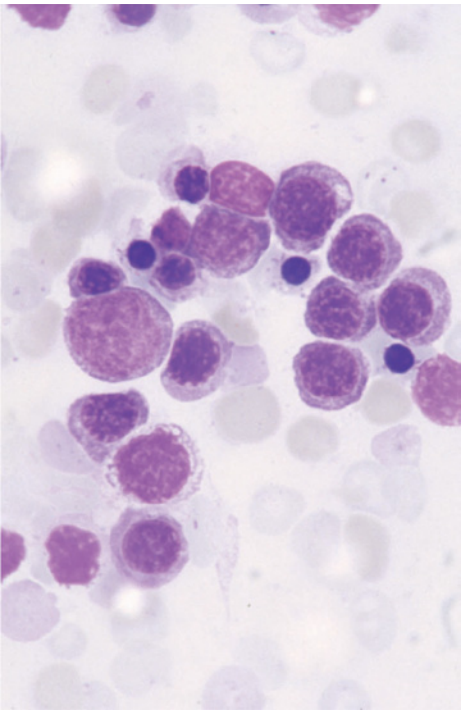
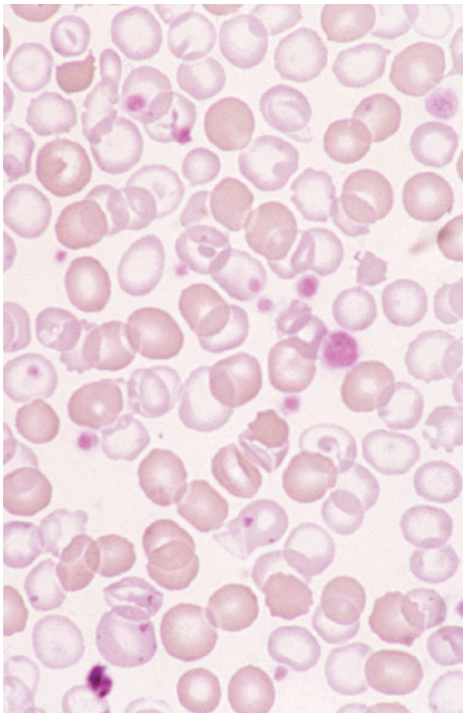
What abnormalities are shown by the blood and bone marrow films?

.....

Explain the nature of the patient's condition.

.....

(With thanks to Dr Jane Mercieca)



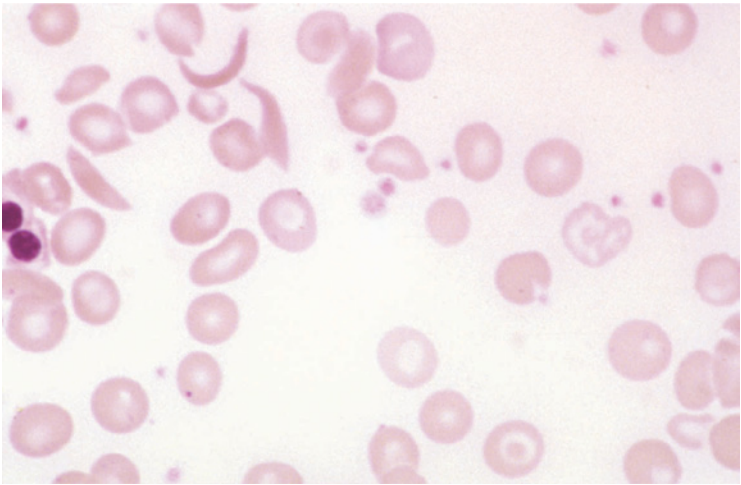
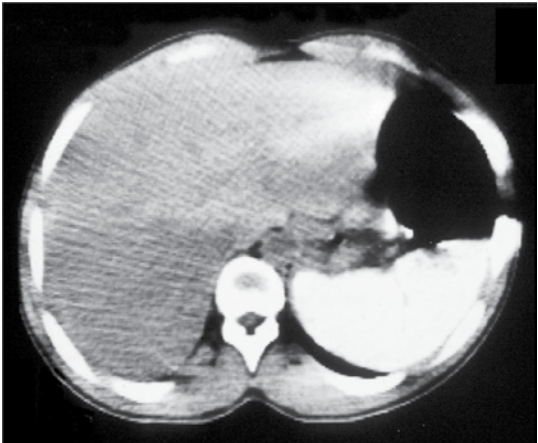
Exercise 8.14

You are provided with photographs of a blood film and a computed tomography (CT) scan of the abdomen performed without any contrast medium, from a young African man with sickle

cell anaemia. The blood film also showed Howell-Jolly bodies.

Explain the blood film and CT scan features in relation to each other.

.....
.....

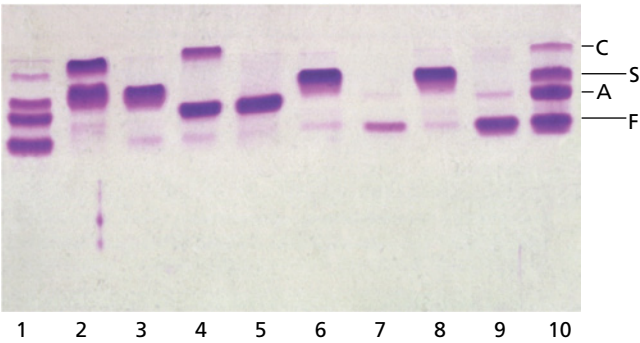
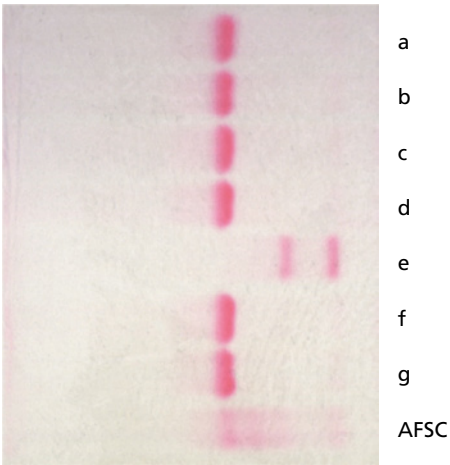
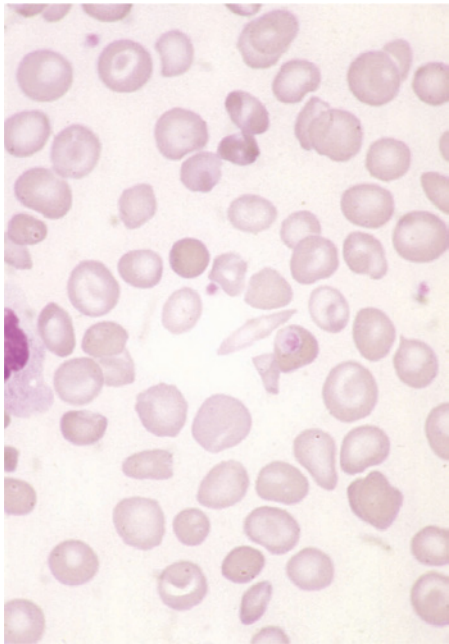


Exercise 8.15

You are provided with photographs of a blood film, haemoglobin electrophoresis on cellulose acetate at alkaline pH (lane e) and haemoglobin

electrophoresis on agarose gel at acid pH (lanes 6 and 8) from a young African Caribbean woman with anaemia and recurrent limb pains.

What is the most likely diagnosis?
.....



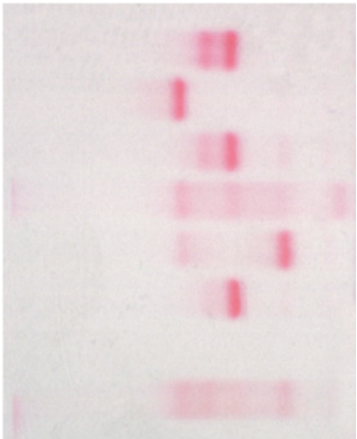
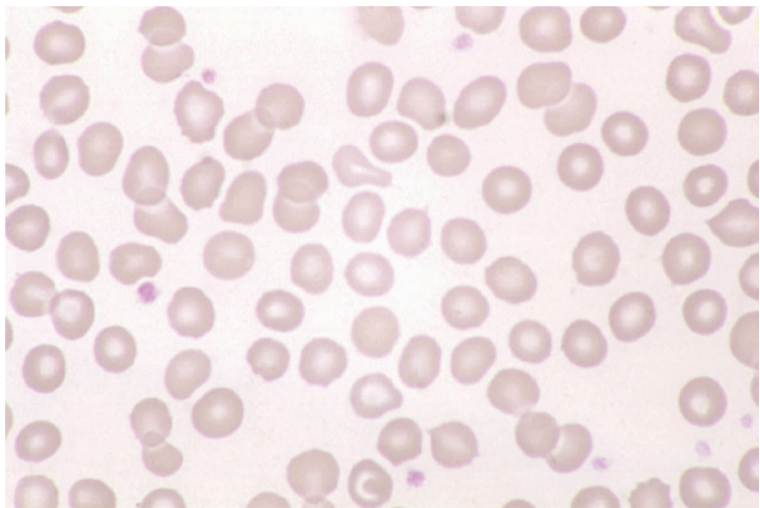
Exercise 8.16

You are provided with photographs of a blood film and haemoglobin electrophoresis on cellulose acetate at alkaline pH (sixth lane) from an African woman who has been hospitalised with a breast abscess. Red cell indices were: RBC $4.23 \times 10^{12}/l$, Hb 107 g/l, MCV 75 fl, MCH 25.3 pg, MCHC 335 g/l and RDW 15.3%. A sickle solubility test was positive. Haemoglobin electrophoresis on agarose gel at acid pH showed two major bands with the mobility of A and S.

What is the most likely diagnosis?

What is the significance of this diagnosis?

What are the possible explanations of the microcytosis?



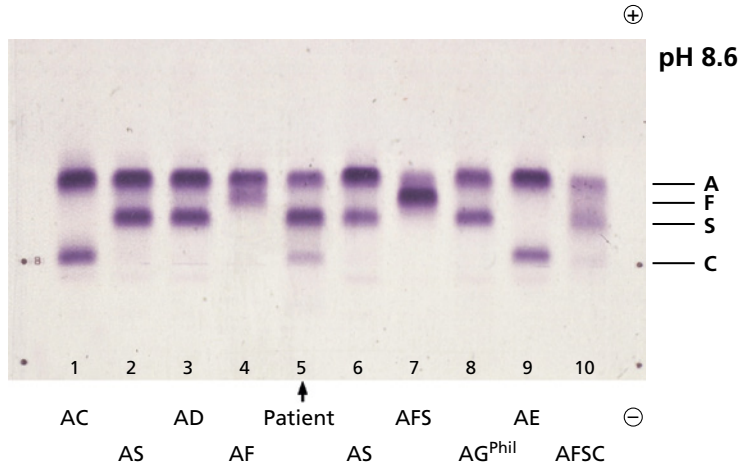
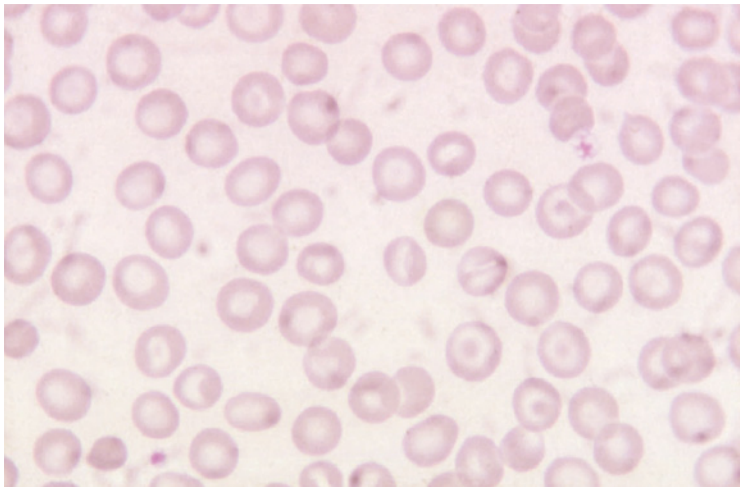
FS
A
FS
AG^{Phil} C hybrid
AC
Patient
AFSC

Exercise 8.17

You are provided with photographs of the blood film and haemoglobin electrophoresis at alkaline pH (fifth lane from left) of an African Caribbean woman in the first trimester of pregnancy. On agarose gel at acid pH there were two

bands with the mobilities of A and S. The sickle solubility test was positive.

What is the most likely diagnosis?
.....
What is the significance to the patient?
.....



Exercise 8.18

A 32-year-old pregnant Chinese woman had normal haemoglobin electrophoresis (haemoglobin A₂ 2.1%) and the following red cell indices: RBC $5.71 \times 10^{12}/l$, Hb 115 g/l, Hct 0.38, MCV 66 fl, MCH 20.1 pg and MCHC 304 g/l.

Exercise 8.19

You are provided with a photograph of the blood film of an African Caribbean woman in the first trimester of pregnancy. Her red cell indices were: RBC $4.2 \times 10^{12}/l$, Hb 120 g/l, MCV 88 fl, MCH 28.6 pg and MCHC 324 g/l. She was found to be Rh D negative with no atypical

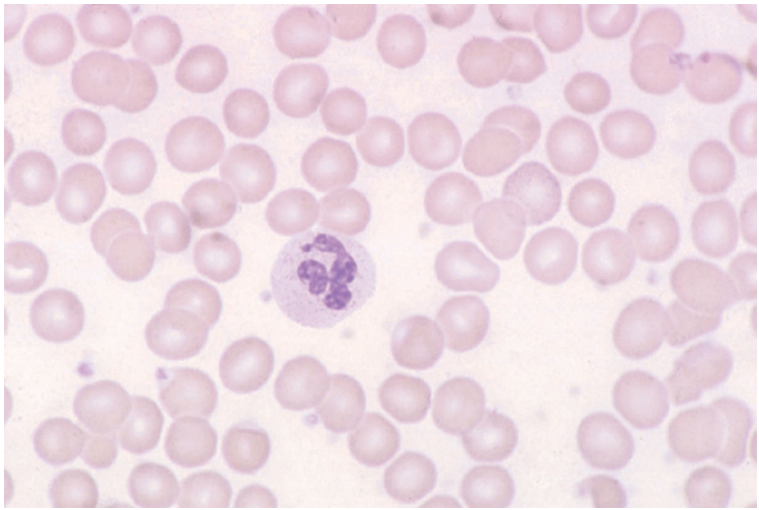
What diagnoses are likely?

What tests should be performed in her partner and why?

antibodies. Haemoglobinopathy screening showed haemoglobins A, F and A₂ with haemoglobin F being 23% of total haemoglobin and haemoglobin A₂ 1.6%.

What is the most likely diagnosis?

What are the implications for management of the patient?



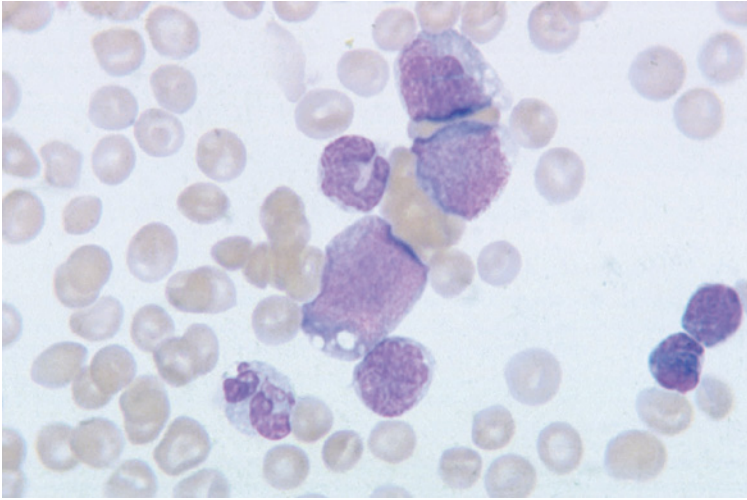
Exercise 8.20

You are provided with a photograph of the blood film of a two-year-old northern European boy who was noted by his parents to be miserable and fretful. On examination, he had hepatosplenomegaly, moderate enlargement of lymph nodes and an eczematous rash. There was a family history of neurofibromatosis and the child himself was noted to have occasional

café-au-lait spots. Results of FBC were: WBC $28.3 \times 10^9/l$, Hb 84 g/l, MCV 78 fl and platelet count $96 \times 10^9/l$. Monocytes and neutrophils were both increased. Haemoglobin electrophoresis showed 12% haemoglobin F and 1.0% haemoglobin A₂. The carbonic anhydrase band on the stained electrophoretic strip appeared to be reduced.

What is the likely diagnosis?

.....



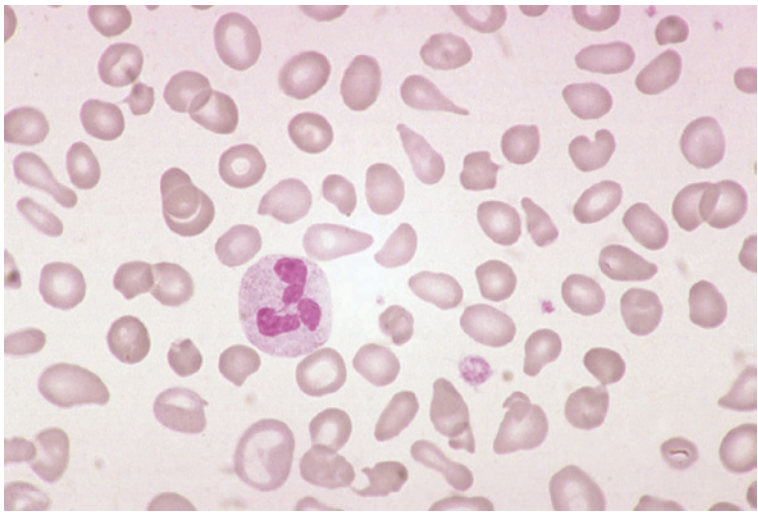
Exercise 8.21

You are provided with a photograph of the blood film of a 17-year-old girl with a white English mother and a Pakistani father. She had been found to be anaemic when she required extraction of wisdom teeth. There was no hepatomegaly

or splenomegaly. The red cell indices and results of haemoglobin A₂ quantification in the girl and her parents are tabulated.

Suggest reasons that might explain why the girl is more anaemic than her father.
(With thanks to Dr Michael Makris)

	Propositus	Father	Mother
RBC ($\times 10^{12}/l$)	4.04	5.99	4.22
Hb (g/l)	85	120	136
MCV (fl)	68.8	62.9	93.1
MCH (pg)	21.0	20.2	32.2
Haemoglobin A ₂	4.7	4.4	2.3
Haemoglobin F	3.2	<1.0	<1.0

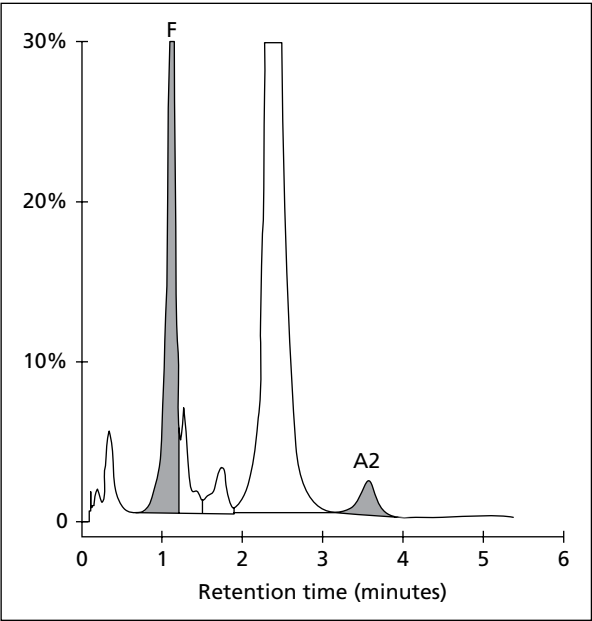


Exercise 8.22

You are provided with an HPLC chromatogram (Bio-Rad Variant) of a patient with the following red cell indices: RBC $4.56 \times 10^{12}/\text{l}$, Hb 113 g/l, Hct 0.34, MCV 75 fl, MCH 24.7 pg and MCHC 332 g/l.

Haemoglobin F was 18.3% and haemoglobin A₂ was 2.6% by HPLC and 1.8% by microcolumn chromatography.

What is the likely diagnosis?



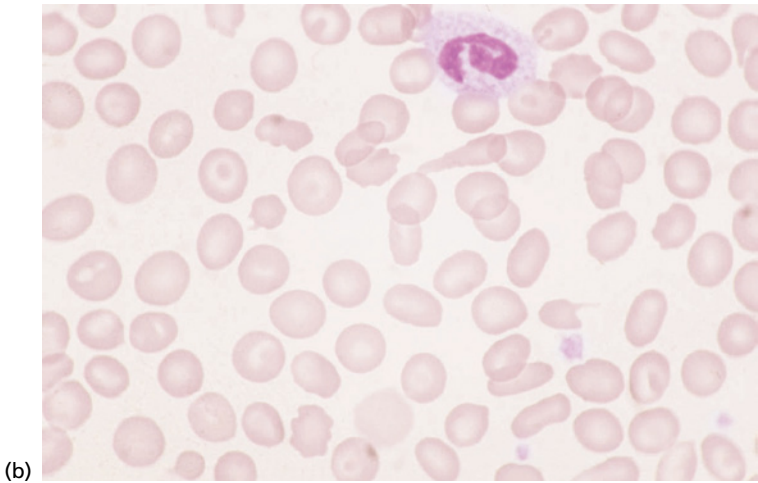
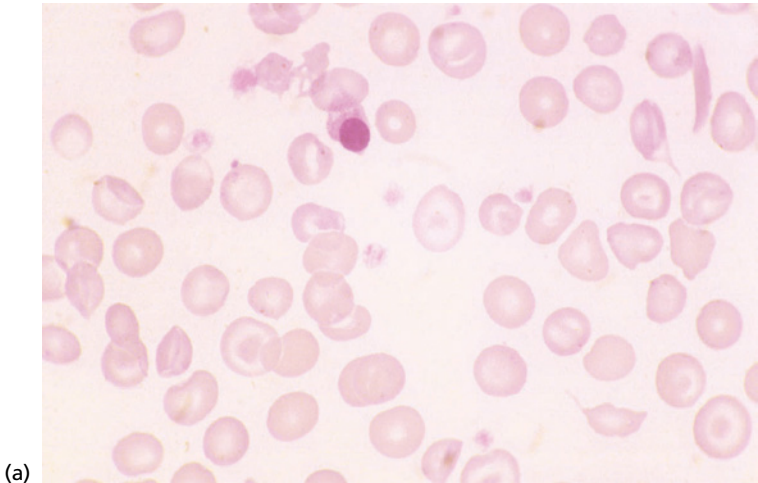
Exercise 8.23

You are provided with photographs of blood films of a girl aged 18 (a) and her mother (b). The girl's father had a normal blood film. Other details are tabulated.

What is the most likely diagnosis in the mother?

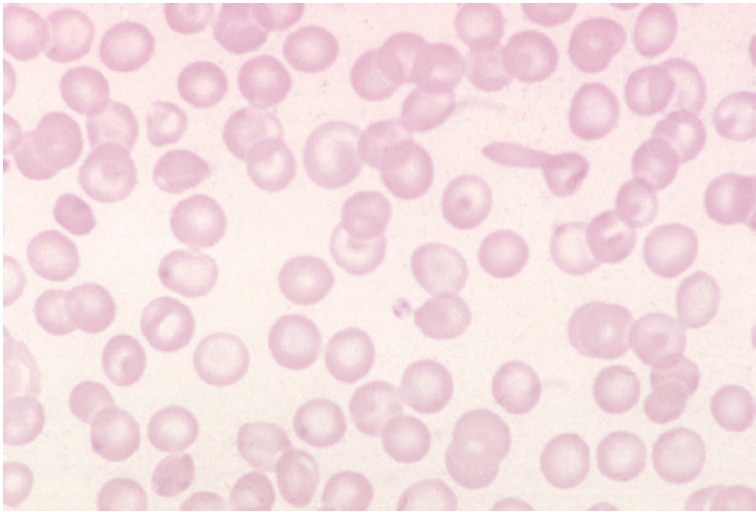
What is the most likely diagnosis in the daughter?

	Propositus	Mother	Father
Ethnic origin	Italian-African	Italian	West African
RBC ($\times 10^{12}/l$)	3.65	5.78	5.1
Hb (g/l)	70	105	153
MCV (fl)	60	56	90
MCH (pg)	19.2	18.2	30
MCHC (g/l)	319	323	326
Ferritin ($\mu\text{mol}/l$)	430	33	50



Exercise 8.24

You are provided with a photograph of the blood film of a patient with the following red cell indices: RBC $5.39 \times 10^{12}/\text{l}$, Hb 126 g/l, Hct 0.37, MCV 69 fl, MCH 23.4 pg and MCHC 340 g/l.



Haemoglobin electrophoresis showed haemoglobin A 23%, haemoglobin S 73% and haemoglobin A₂ 4%.

What is the most likely diagnosis?
.....

Exercise 8.25

A three-year-old English boy was brought to the Paediatric Accident and Emergency Department by his father who had found him playing in the garden shed several hours previously. The child had complained of abdominal pain and had vomited. Thereafter he appeared restless and lethargic. On examination in the emergency department, he was noted to have central cyanosis, tachycardia, tachypnoea and hypotension. He was afebrile. Heart sounds were normal and there was no abnormality on auscultation of the lungs. Arterial blood gas

analysis showed a normal partial pressure of oxygen and reduced partial pressure of CO₂. An FBC showed WBC $14.3 \times 10^9/\text{l}$, Hb 110 g/l, MCV 85 fl, MCH 27 pg and platelet count $360 \times 10^9/\text{l}$. The laboratory scientist performing the blood count and blood gas analysis noted that the blood was chocolate-brown in colour.

What is the most likely diagnosis?
.....

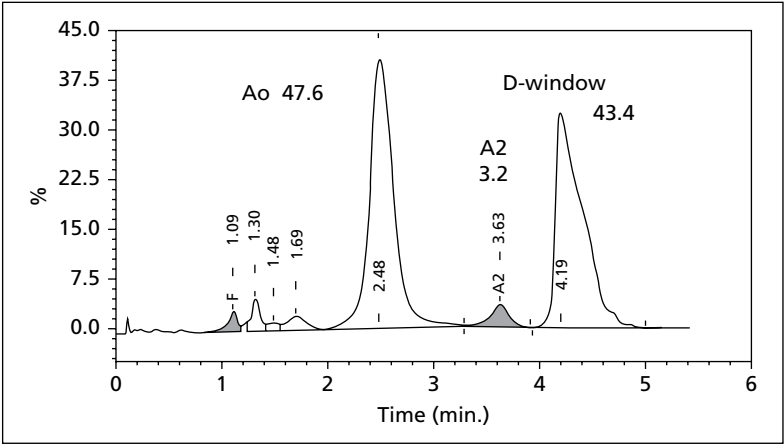
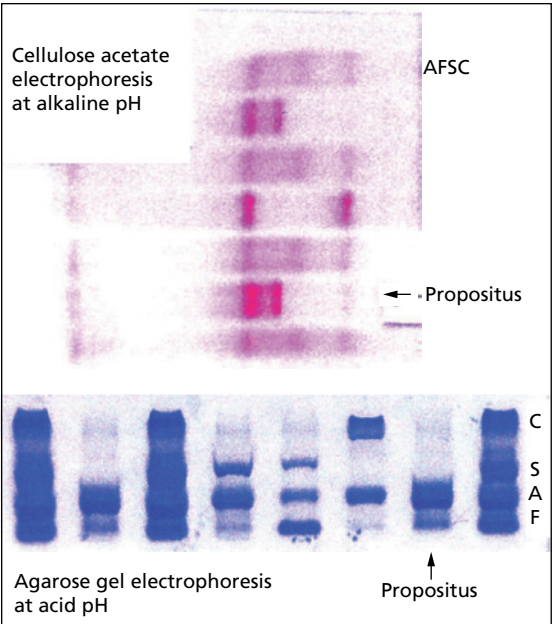
What test should be done?
.....

What treatment should be given?
.....

Exercise 8.26

You are provided with results of cellulose acetate electrophoresis at alkaline pH, agarose gel electrophoresis at acid pH and an HPLC chromatogram (Bio-Rad Variant II) on an Irish woman known to have had a high haemoglobin concentration for some time. On the agarose gel electrophoresis, the patient's blood sample is

second from the right between an AC sample (to the left) and an FASC control sample (to the right). Her WBC and platelet count were normal. Red cell indices were: RBC $4.71 \times 10^{12}/l$, Hb 161 g/l, Hct 0.46, MCV 96.8 fl, MCH 34.1 pg, MCHC 352 g/l and RDW 12.2%.
What is the likely diagnosis?
(With thanks to Mairead O'Reilly)



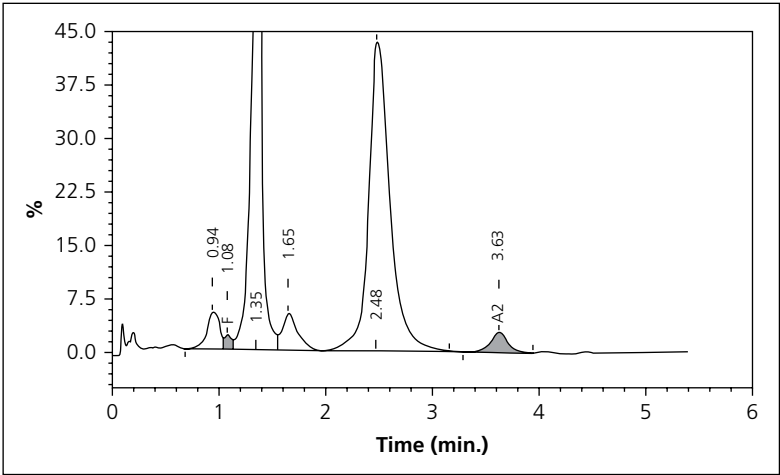
Exercise 8.27

You are provided with an HPLC chromatogram (Bio-Rad Variant II) on an anaemic pregnant African woman (Hb 98 g/l, MCV 79 fl). The 'P2 fraction' on Bio-Rad variant II HPLC analysis was 41.4% with the abnormal peak having a retention time of 1.35 minutes. On cellulose

acetate electrophoresis at alkaline pH, the A band was broadened by a slightly faster component. Agarose gel electrophoresis at acid pH was normal.

Is this likely to indicate pregnancy-related diabetes or is there an alternative explanation?

.....



Exercise 8.28

Three previously healthy children whose father was a butcher became profoundly cyanosed, with a deteriorating level of consciousness, within a short time of each other. Rapid recov-

ery occurred following intravenous injection of methylene blue.

What is the likely cause of the cyanosis?
.....

What is the probable underlying cause?
.....

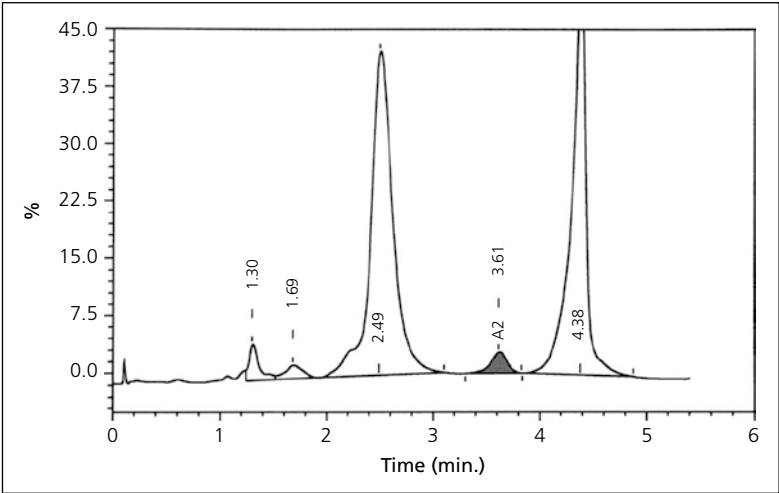
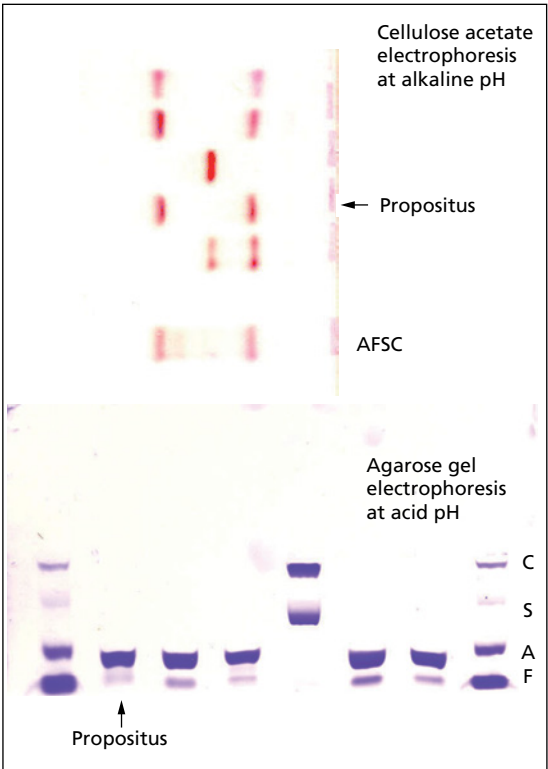
Exercise 8.29

The blood of a 34-year-old pregnant woman from northern Europe was sent for antenatal thalassaemia and haemoglobinopathy screening in a hospital with a policy of universal screening. Her red cell indices were: RBC $4.23 \times 10^{12}/\text{l}$, Hb 134 g/l, Hct 0.39, MCV 92 fl, MCH 31.6 pg and MCHC 346 g/l. You are provided with cellulose acetate electrophoresis at

alkaline pH and agarose gel electrophoresis at acid pH.

Make a provisional diagnosis.....

After reaching a provisional diagnosis, examine the HPLC chromatogram (Bio-Rad Variant II) provided and assess whether the provisional diagnosis is confirmed. State the significance of your findings. The variant haemoglobin was 41.9% and had a retention time of 4.38 minutes.
.....

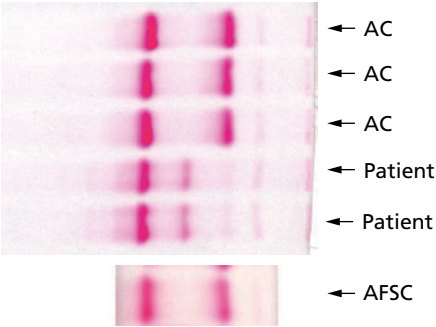
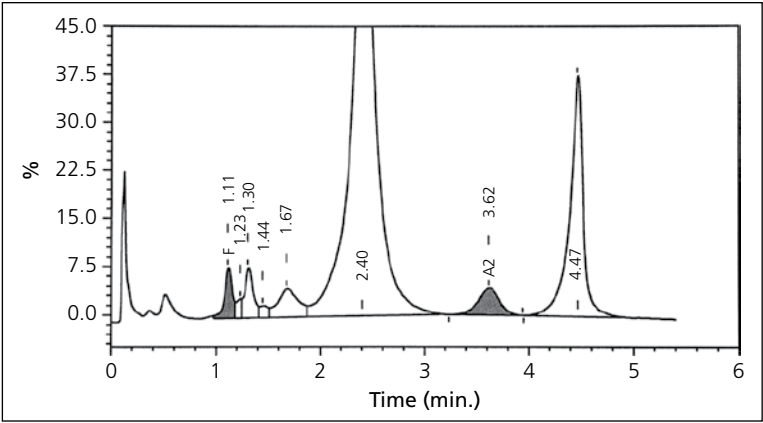


Exercise 8.30

Haemoglobinopathy investigation was requested on a 15-year-old boy hospitalised with vomiting and diarrhoea. His ethnic origin was not totally clear; he was stated to be 'black' and had a name that suggested he might be Arab or from a Muslim country. Red cell indices were: RBC $5.18 \times 10^{12}/l$, Hb 86 g/l, Hct 0.286, MCV 55 fl, MCH 16.6 pg, MCHC 300 g/l and RDW 24%. His blood film showed hypochromia, microcytosis, numerous target cells, basophilic stippling and

some irregularly contracted cells. You are provided with haemoglobin electrophoresis on cellulose acetate at alkaline pH and an HPLC chromatogram (Bio-Rad Variant II) (done on washed cells). The smaller peak on the right-hand side of the chromatogram was in the S window and was 20%; a sickle solubility test was positive. Haemoglobin F was 3% and haemoglobin A₂ was quantified as 4%.

Explain your findings and give a provisional diagnosis.....



Exercise 8.31

Preoperative haemoglobinopathy screening was requested in a 26-year-old woman. She was found to have a haemoglobin A₂ of 5.3% and

haemoglobin F of 2.5%. Her red cell indices were: RBC $4.64 \times 10^{12}/l$, Hb 129 g/l, Hct 0.41, MCV 89 fl, MCH 27.7 pg and MCHC 312 g/l.

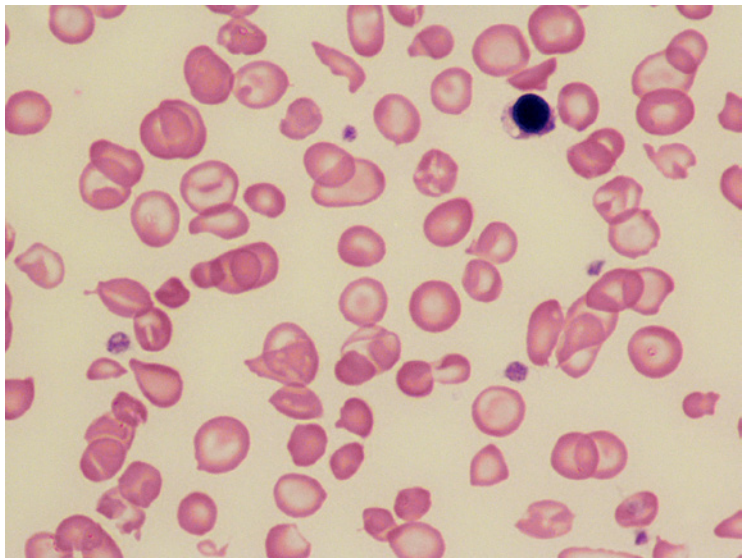
Does she have β thalassaemia trait?
How would you proceed?

Exercise 8.32

A 13-year-old Ghanaian boy had splenomegaly and tired easily. One brother was similarly affected while another had a milder anaemia. His FBC showed: RBC $5.32 \times 10^{12}/l$, Hb 85 g/l, Hct 0.30, MCV 57 fl, MCH 16.1 pg and MCHC

281 g/l. You are provided with a photograph of his blood film. HPLC showed haemoglobin F 96.7% and haemoglobin A₂ 3.3%.

What abnormalities are shown in the blood film?
What is the most likely diagnosis?

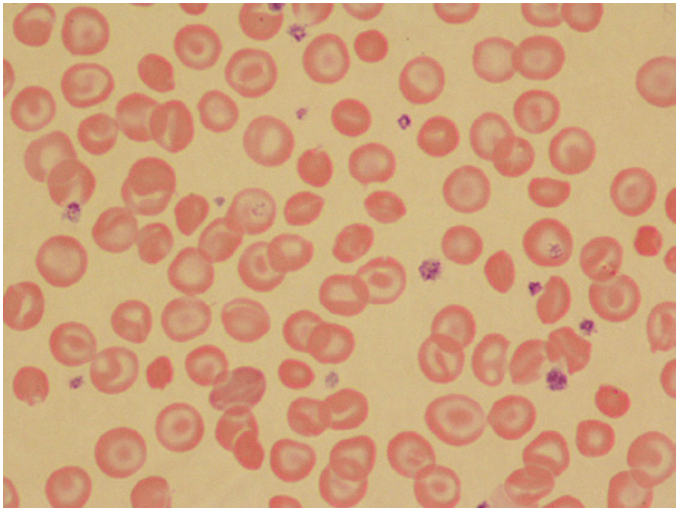


Exercise 8.33

A 21-month-old infant was being followed in Paediatric Haematology outpatients after detection of an abnormality on neonatal haemoglobinopathy screening. She was clinically well. Her FBC (with age-appropriate reference ranges in brackets) showed Hb 92 g/l (105–135), MCV 57.8 fl (70–85 fl), MCH 20.7 pg (25–35 pg), MCHC 358 g/l (310–350 g/l) and RDW 16.3% (12.5–15.5%). Serum iron was 5 μ mol/l, transferrin

2.6 g/l and transferrin saturation 8%. The infant's mother was of Thai ethnic origin and her father was African Caribbean. You are provided with photographs of a blood film and an HPLC chromatogram (Bio-Rad Variant II).

What abnormalities are shown by the blood film?
Considering the ethnic origin, what are the most likely diagnoses?.....
Comment on the proportions of the variant haemoglobins.....



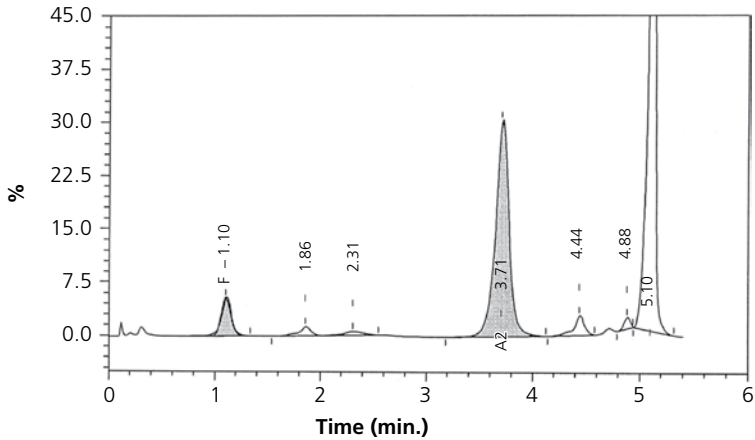
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	4.6*	---	1.10	80295
P3	---	1.3	1.86	25215
Ao	---	0.9	2.31	17335
A2	30.4*	---	3.71	633239
S-window	---	2.3	4.44	44767
Unknown	---	0.7	4.88	14447
C-window	---	57.8	5.10	1114765

Total Area: 1,930,063

F Concentration = 4.6*%
A2 Concentration = 30.4*%

*Values outside of expected ranges

Analysis comments:



Exercise 8.34

A blood sample from a 31-year-old woman complaining of tiredness was sent for an FBC and haemoglobinopathy investigations by her general practitioner. This showed RBC $5.0 \times 10^{12}/l$, Hb 99 g/l, Hct 0.33, MCV 66 fl, MCH 20.1 pg, MCHC 300 g/l and RDW 20%.

Her ethnic origin had not been disclosed but her name suggested that she was not northern European. You are provided with her HPLC chromatogram (Bio-Rad Variant II).

What is the most likely diagnosis?

What is the most likely ethnic origin?

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.2	0.99	2071
F	0.5	---	1.08	5962
Unknown	---	0.6	1.22	7090
P2	---	2.7	1.30	34591
P3	---	2.1	1.68	26598
Ao	---	92.7	2.47	1169988
A2	1.4*	---	3.62	15744

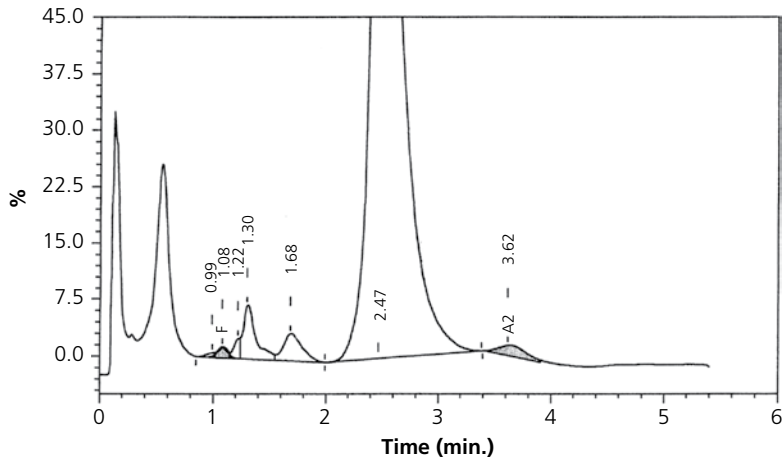
Total Area: 1,262,044

F Concentration = 0.5%

A2 Concentration = 1.4*%

*Values outside of expected ranges

Analysis comments:



Exercise 8.35

You are provided with an HPLC chromatogram (Bio-Rad Variant II) on an adult patient with a

normal blood count and a positive sickle solubility test.

What diagnoses can be made in this patient?

.....

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.69	2767
Unknown	---	0.1	0.96	1310
F	3.0*	---	1.20	54810
P2	---	8.3	1.29	156107
P3	---	4.8	1.68	90436
Ao	---	50.2	2.51	947221
A2	3.9*	---	3.64	69107
S-window	---	30.0	4.48	566796

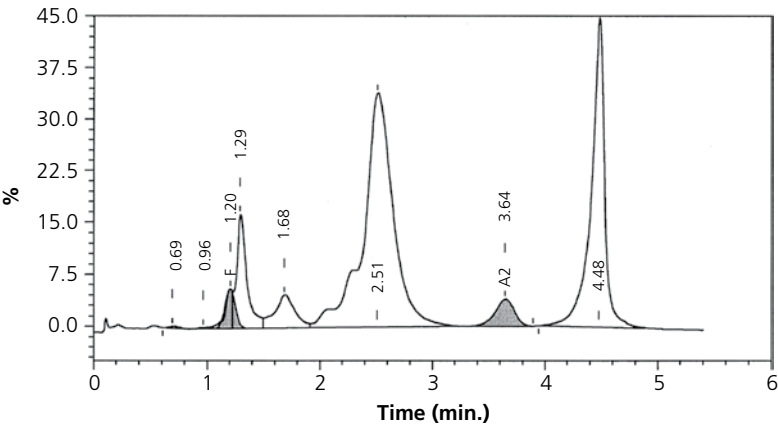
Total Area: 1,888,555

F Concentration = 3.0*%

A2 Concentration = 3.9*%

*Values outside of expected ranges

Analysis comments:



Exercise 8.36

A pregnant African Caribbean woman was known to have β thalassaemia trait and this was confirmed on her presentation to the antenatal clinic in her second pregnancy. Her partner was tested and found to have an Hb of 130 g/l, MCV 58 fl and MCH 17.8 pg. HLPC (Bio-Rad Variant II) showed haemoglobin A₂ 3% and a peak in the haemoglobin S window of 2.5%. No further investigations were done.

At five months of age the baby was found to have anaemia (Hb 80 g/l), hepatosplenomegaly and growth retardation. HPLC showed haemoglobin A 51.5%, haemoglobin F 46% and haemoglobin A₂ 2.5%.

What has gone wrong?

Exercise 8.37

The following is an extract from the Manchester Guardian of 30th September, 1902.

**TRAGIC DEATH OF E. ZOLA
ACCIDENTALLY ASPHYXIATED
MADAME ZOLA NEARLY SHARES HIS
FATE**

We regret to announce the death, under circumstances of a most tragic character, of the renowned French novelist, M. Emile Zola. The following telegram explains how M. Zola died: -

Paris

M. Emile Zola was this morning found dead in his house from accidental asphyxiation. Madame Zola is seriously ill.

2.00 p.m. The death of M. Zola appears to have been caused by poisonous gases emitted from a stove, the pipe of which is stated to have fitted badly. It is believed that doctors will be able to save the life of Madame Zola, who was also affected by the noxious vapour.

From such details as have hitherto been obtained it seems that M. and Madame Zola returned yesterday from the country where they had been staying for about three months. Their house in Rue de Brielle's was very cold, not having been inhabited for so long, and as there was a considerable fall in the temperature, M. Zola ordered the fire to be lighted in the grate of the bedroom, which is a vast apartment. The footman set about lighting the fire, but it did not draw at all well. After dinner, which M. and Madame Zola ate with good appetite they retired to rest. That was about ten o'clock.

This morning at 9.30 a.m. some workmen went to the house to execute certain repairs which had been ordered to be carried out in M. Zola's room. The servants, who were already a little alarmed at having heard no sound in the bedroom, knocked loudly at the door, which, as they received no response, they broke in ... M. Zola was found lying half out of bed ... He was quite dead. Madame Zola was found lying in bed showing no signs of life ... The frightened servants instantly threw open the windows and gave the alarm.

What is the 'noxious vapour' to which death is attributed?

Explain the mechanism of death.....

Did the servants take the right action?
.....

Answers

Exercise 8.1

Patient 1: AS.

Patient 2: AD or AG (heterozygosity for a number of haemoglobins designated D or G, both α and β chain variants, for example D-Punjab, D-Iran, D-Copenhagen, D-Norfolk or G-Philadelphia).

Note: the quantity of the variant and haemoglobin A are similar so a β chain variant is favoured.

Patient 3: A plus Lepore.

Patient 4: SS or S β^0 thalassaemia.

Patient 5: SD (e.g. S plus D-Punjab since the proportions are equal and a β chain variant is favoured).

Patient 6: AS plus G-Philadelphia.

Patient 7: AD plus G-Philadelphia (i.e. heterozygosity for a β chain variant designated D or G, e.g. D-Punjab, and an α chain variant designated D or G, e.g. G-Philadelphia).

Patient 8: AC.

Patient 9: AE (note that the quantity of the variant haemoglobin is less than in C trait and, in addition, the mobility at acid pH is different).

Patient 10: A plus C-Harlem.

Patient 11: A plus O-Arab.

Exercise 8.2

Patient 1: AS (sickle cell trait).

Patient 2: A plus D-Punjab (the HPLC chromatogram is typical of D-Punjab heterozygosity).

Exercise 8.3

Haemoglobin E trait is most likely because of the ethnic origin, the thalassaemic indices, the mobility of the variant haemoglobin at alkaline pH and the relatively low percentage of the variant haemoglobin (the variant plus haemoglobin A₂ appears to be around 30%). Haemoglobin C and haemoglobin O-Arab are much less likely. The provisional diagnosis could be confirmed by haemoglobin electrophoresis at acid pH, by HPLC or by capillary electrophoresis.

The condition of most potential significance in the partner would be β thalassaemia trait since the compound heterozygous state for haemoglobin E and β thalassaemia often leads to the clinical picture of thalassaemia intermedia or even thalassaemia major.

Exercise 8.4

The findings are indicative of heterozygosity for $\delta\beta$ thalassaemia since there are thalassaemic indices with elevation of haemoglobin F but not haemoglobin A₂. It would be sensible to test the patient's father and, if he were normal, the patient's mother, to help confirm the diagnosis.

The patient's father was tested and was found to have the same haematological abnormality as the patient. The clinical significance is very similar to the clinical significance of β thalassaemia heterozygosity. The patient should not take iron unless she is demonstrated to be iron deficient and should be warned of the possibility of thalassaemia intermedia or major in the fetus if her partner should happen to be heterozygous for β (or $\delta\beta$) thalassaemia. If she were to become pregnant it should be noted that her high fetal haemoglobin percentage would be expected to result in a positive Kleihauer test. If she were Rh D negative she would need an alternative test for detection of fetomaternal haemorrhage.

Exercise 8.5

There is a band with the mobility of S at alkaline pH, which we can deduce had the same mobility as A at acid pH. On the HPLC chromatogram it is seen that the variant haemoglobin is eluting in the haemoglobin A₂ window and, together with haemoglobin A₂, comprises 14.3% of total haemoglobin. Clearly, it is not haemoglobin A₂ on the grounds of the proportion of the variant and its electrophoretic mobility. These findings are indicative of haemoglobin Lepore trait.

The significance is similar to that of β thalassaemia trait.

For a bonus point you might have noted that the P2 fraction eluting after haemoglobin F, representing glycosylated haemoglobin, is also quite high.

Exercise 8.6

Father: β thalassaemia trait plus hereditary elliptocytosis.

Daughter aged 17: hereditary elliptocytosis.

Daughter aged 15: β thalassaemia trait.

Son aged 10: normal.

The father's blood film shows anisocytosis, poikilocytosis including elliptocytes and ovalocytes, microcytosis and hypochromia. There is a teardrop poikilocyte showing basophilic stippling. His haemoglobin A₂ is elevated. The film is more strikingly elliptocytic than is usual in β thalassaemia trait and consideration of the conditions he has transmitted to two of his children indicates that he is likely to have both β thalassaemia trait and hereditary elliptocytosis.

Exercise 8.7

The clinical history and initial laboratory findings suggest a diagnosis of iron deficiency anaemia. The subsequent demonstration of a variant haemoglobin, identified as haemoglobin S, is likely to be correct since it was based on electrophoresis at acid and alkaline pH and HPLC (although a positive sickle solubility test would not be expected). An explanation needs to be found for haemoglobin S being present in such a low concentration. There are two possible explanations.

- She has been transfused with blood from a donor with sickle cell trait.
- She herself has sickle cell trait and the percentage of the variant haemoglobin has been lowered by the recent transfusion.

It is unlikely that the patient has sickle cell disease since there is nothing in the history to suggest this. The fact that she is identified as 'white' does not exclude her having haemoglobin S. However, haemoglobin electrophoresis on a residual blood sample from each of the donor bags confirmed that the patient had been transfused with blood from a donor with sickle cell trait.

It is not rare for clinical staff to request investigation for a haemoglobinopathy when there is no clear clinical indication for the test nor is it rare for a test to be requested, inappropriately, on a blood sample taken *after* transfusion. The laboratory should be alert for this explanation of anomalous results. Transfusion of blood from a donor with haemoglobin C has also been reported.

Ahmad E and Sykes E (1999) Clinical pathology rounds: low level of hemoglobin S in a white woman. *Lab Med*, **30**, 572–575.

Suarez AA, Polski JM, Grossman BJ and Johnston M (1999) Blood transfusion-acquired hemoglobin C. *Arch Path Lab Med*, **123**, 642–643.

Exercise 8.8

The blood film shows target cells, irregularly contracted cells and a haemoglobin C crystal. Electrophoresis on cellulose acetate at alkaline pH shows only haemoglobin C but on agarose gel faint bands with the mobilities of F and A are also present, in addition to the major C band. A laboratory error might be suspected as

the explanation of this discrepancy but in fact, agarose gel electrophoresis is more sensitive than cellulose acetate electrophoresis for the detection of a low concentration of normal and variant haemoglobins. Globin chain synthesis studies confirmed that the patient was a compound heterozygote for β^C and β^+ thalassaemia with greatly reduced synthesis of β^A globin chain.

Exercise 8.9

The mother has D or G trait. The father has sickle cell trait. The child obviously has a sickling disorder and must have inherited D from the mother and S from the father. The blood film of the child indicates that there has been interaction between haemoglobin S and the other variant haemoglobin, producing a clinically significant disorder. This is likely to be haemoglobin S/haemoglobin D-Punjab compound heterozygosity. Other haemoglobins designated D and G do not interact adversely with haemoglobin S. Haemoglobin D-Punjab was confirmed on further testing.

This case shows the importance of performing HPLC, capillary electrophoresis or electrophoresis at acid pH in patients who, on cellulose acetate electrophoresis, have a single band with the mobility of haemoglobin S. Misdiagnosis of compound heterozygous states as sickle cell anaemia may lead to paternity being questioned, in cases in which the father does not have haemoglobin S, with serious social and possibly legal consequences.

The situation is more straightforward if HPLC or capillary electrophoresis is the initial test, rather than cellulose acetate electrophoresis, since S and D-Punjab can then be distinguished (see Figs 5.34 and 5.35).

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	15.2*	---	1.10	124732
P2	---	0.3	1.34	2515
P3	---	0.2	1.75	1568
Ao	---	2.0	2.12	18157
Unknown	---	0.8	2.29	7234
Unknown	---	1.7	2.53	15505
A2	3.9*	---	3.65	38053
D-window	---	42.5	4.07	384769
S-window	---	34.5	4.33	311901

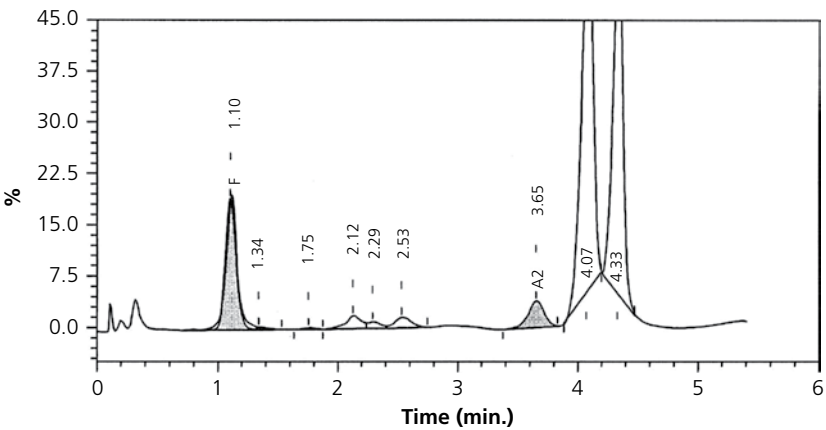
Total Area: 904,433*

F Concentration = 15.2* %

A2 Concentration = 3.9*%

*Values outside of expected ranges

Analysis comments:



HPLC chromatogram (Bio-Rad Variant II) in another patient with haemoglobins S and D-Punjab. The peaks from left to right are altered haemoglobin F, haemoglobin F₀ (shaded), post-translationally modified haemoglobin S in the A₀ window, haemoglobin A₂, haemoglobin S and haemoglobin D-Punjab.

Exercise 8.10

Patient 1: SC.

Patient 2: SE.

Patient 3: S plus C-Harlem or SS plus G-Philadelphia (or other D/G α chain variant).

Patient 4: S plus O-Arab.

Patient 5: CC plus G-Philadelphia.

Patient 6: AC plus G-Philadelphia.

Patient 7: SC plus G-Philadelphia.

Patient 8: CC or C β^0 thalassaemia.

Patient 9: EE or E β^0 thalassaemia.

Patient 10: O-Arab homozygote or O-Arab/ β^0 thalassaemia.

Patient 11: C plus C-Harlem.

Exercise 8.11

The electrophoretic strip shows only haemoglobin A₂ and a variant haemoglobin that is slightly faster than haemoglobin A. There is no common variant haemoglobin with this mobility. The two possible explanations are:

- compound heterozygosity for β^0 thalassaemia and an uncommon variant haemoglobin
- homozygosity for an uncommon haemoglobin.

In the absence of consanguinity it is unlikely that the patient would be homozygous for an uncommon variant haemoglobin. DNA analysis confirmed that he was heterozygous for β^0 thalassaemia, and electron spray mass spectrometry (with thanks to Dr Barbara Wild) identified the variant haemoglobin as haemoglobin Tacoma.

As these investigations are said to have resulted from a 'routine blood count', it appears that the patient is asymptomatic (this was confirmed) and the variant haemoglobin is therefore not likely to be of any clinical significance. The β thalassaemia trait, however, could have genetic significance.

Exercise 8.12

The patient has sickle cell trait since she has a positive sickle solubility test and a variant haemoglobin with the mobility of haemoglobin S. However, the haemoglobin S percentage is unusually low. This, together with the thalassaemic indices, suggests that as well as having sickle cell trait, she is homozygous for α^+ thalassaemia. The genotype $-\alpha/-\alpha$ is found in 1–2% of African Caribbeans. The genotype $--/\alpha\alpha$ is

very rare in this ethnic group so is a much less likely explanation. The S band is so faint that, except for the positive sickle solubility test, haemoglobin Lepore might have been suspected.

Exercise 8.13

The blood film is dimorphic and shows target cells, Pappenheimer bodies and giant platelets. The bone marrow shows marked erythroid hyperplasia. Inherited haemoglobin H disease is unlikely in view of the ethnic origin and the presence of haematological abnormalities indicative of a haematological neoplasm. Because blasts are a high percentage of non-erythroid cells, the disease was classified, according to the French-American-British (FAB) classification, as M6 acute myeloid leukaemia (AML) rather than as a myelodysplastic syndrome (MDS). However, as blast cells were only 3.6% of all nucleated cells, the classification, according to the 2016 World Health Organization (WHO) Classification of Tumours of Haemopoietic and Lymphoid Tissues, would not have been as AML. It is of interest that the patient has both myelodysplastic features (anaemia and some ring sideroblasts) and myeloproliferative features (thrombocytosis with giant platelets). This represents an 'overlap syndrome', i.e. a condition with overlapping myelodysplastic/myeloproliferative features, recognised as such in both the 2016 and 2022 WHO classifications. This patient illustrates the particular association of acquired haemoglobin H disease with a myeloid neoplasm with prominent erythroid involvement.

Bain BJ (1999) The relationship between the myeloproliferative syndromes and the myeloproliferative disorders. *Leuk Lymphoma*, **34**, 443–449.

Mercieca J, Bain B, Barbour G and Catovsky D (1996) Teaching cases from the Royal Marsden and St Mary's Hospitals Case 10 Microcytic anaemia and thrombocytosis. *Leuk Lymphoma*, **21**, 185–186.

Exercise 8.14

The blood film shows that the patient has the features expected in sickle cell anaemia, including hyposplenic features (a target cell and a Howell-Jolly body). The CT scan shows that rather than having splenic atrophy, the patient's spleen is of normal size

and abnormally dense. This unusual appearance suggests that there is deposition of calcium in the spleen, as a result of recurrent splenic infarction. Despite the normal-sized spleen the patient has functional hyposplenism.

Exercise 8.15

It is clear from the history and the blood film that the patient has some type of sickle cell disease. Haemoglobin electrophoresis at alkaline pH suggests possible compound heterozygosity for haemoglobins S and C. However, at acid pH it is clear that there is no haemoglobin C present. Compound heterozygosity for haemoglobin S and C-Harlem should be suspected (and was the answer given in the first edition of this book)*. However, further investigation including family studies, citrate agar electrophoresis and mass spectrometry led to a diagnosis of compound heterozygosity for haemoglobin S and haemoglobin O-Arab. The variable mobility of haemoglobin O-Arab on electrophoresis at acid pH can cause problems in diagnosis of compound heterozygous states. These problems do not arise in the simple heterozygous state since haemoglobin C-Harlem has a positive sickle solubility test and haemoglobin O-Arab does not.

*Haemoglobin O-Arab has been similarly misidentified as haemoglobin C-Harlem by others. HPLC can be useful.

Joutovsky A and Nardi M (2004) Hemoglobin C and hemoglobin O-Arab variants can be diagnosed using the Bio-Rad Variant II high-performance liquid chromatography system without further confirmatory tests. *Arch Pathol Lab Med*, 128, 435–439.

Exercise 8.16

The patient is a compound heterozygote for haemoglobin S and a β chain variant, haemoglobin D or G. Since she is asymptomatic and the blood film does not show any features of sickle cell disease, the second haemoglobin is likely to be a variant that does not interact with haemoglobin S, rather than haemoglobin D-Punjab, which does interact. Although the precise variant was

not identified in this case, it was shown by HPLC not to be haemoglobin D-Punjab.

Only the haemoglobin S heterozygosity is likely to be clinically significant. If the patient requires a general anaesthetic for drainage of the breast abscess, the anaesthetist will wish to know that she has sickle cell trait. This would also be of potential genetic significance.

The anaemia and microcytosis could be caused by the effects of the infection, if it has been going on for some time, leading to anaemia of chronic disease. Alternatively, the patient could have a coincidental iron deficiency anaemia. α thalassaemia trait is also quite likely in this ethnic group.

Exercise 8.17

The patient is heterozygous for both β^S and $\alpha^{G\text{-Philadelphia}}$, hence the three bands on electrophoresis at alkaline pH. The haemoglobin G-Philadelphia is very unlikely to be of any clinical significance. However, if the patient's partner also has sickle cell trait there is a one in four risk of the fetus having sickle cell anaemia. There would also be significant genetic implications if the patient's partner had β thalassaemia trait, haemoglobin C, haemoglobin D-Punjab or haemoglobin O-Arab. The couple concerned might wish to consider termination of pregnancy if significant fetal disease were predicted. Red cell indices and haemoglobin electrophoresis or HPLC should be performed on her partner, followed, if indicated, by consideration of antenatal diagnosis of any significant abnormality in the fetus.

Exercise 8.18

As the patient has marked microcytosis but a normal Hb she is unlikely to have iron deficiency. The red cell indices are suggestive of thalassaemia and as she has a normal haemoglobin A_2 percentage it is likely that she has α thalassaemia. Since she is Chinese she could be heterozygous for α^0 thalassaemia ($--/\alpha\alpha$) or homozygous for α^+ thalassaemia ($-\alpha/-\alpha$). The microcytosis is too marked for heterozygosity for α^+ thalassaemia to be a likely diagnosis. The implications are as shown below.

Abnormality present in mother	Findings in partner	Possible abnormality in fetus
$-\alpha/-\alpha$	$\alpha\alpha/\alpha\alpha$	$-\alpha/\alpha\alpha$ (α thalassaemia trait)
	$-\alpha/\alpha\alpha$	$-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$ (α thalassaemia trait)
	$-\alpha/-\alpha$	$-\alpha/-\alpha$ (α thalassaemia trait)
	$--/\alpha\alpha$	$-\alpha/\alpha\alpha$ (α thalassaemia trait) or $-\alpha/--$ (haemoglobin H disease)
$--/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$ (normal) or $--/\alpha\alpha$ (α thalassaemia trait)
	$-\alpha/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$ (normal), $-\alpha/\alpha\alpha$ (α thalassaemia trait), $--/\alpha\alpha$ (α thalassaemia trait) or $--/-\alpha$ (haemoglobin H disease)
	$-\alpha/-\alpha$	$-\alpha/\alpha\alpha$ (α thalassaemia trait) or $--/-\alpha$ (haemoglobin H disease)
	$--/\alpha\alpha$	$\alpha\alpha/\alpha\alpha$ (normal) or $--/\alpha\alpha$ (α thalassaemia trait) or $--/--$ (haemoglobin Bart's hydrops fetalis)
	β thalassaemia trait	<i>Beware:</i> a diagnosis of β thalassaemia trait in the partner does not exclude his also having α thalassaemia trait; molecular analysis to exclude $--/\alpha\alpha$ is indicated

Exercise 8.19

The high haemoglobin F with normal red cell indices is likely to be caused by deletional hereditary persistence of fetal haemoglobin. This has no clinical significance except that the Kleihauer test will be positive. The patient is Rh D negative and an alternative technique will have to be used after delivery to detect and quantitate any fetal cells in the maternal circulation.

Exercise 8.20

The findings are those of juvenile myelomonocytic leukaemia. An increased haemoglobin F percentage for age is one of the criteria that can be used for making this diagnosis. The low haemoglobin A_2 and low carbonic anhydrase show reversion to fetal type erythropoiesis.

Exercise 8.21

It appears that both the daughter and the father are heterozygous for β thalassaemia since they both have microcytosis and an increased haemoglobin A_2 percentage. This was confirmed on molecular analysis, both having the IVS1 5 G→C mutation. Possible explanations of the more severe phenotype in the daughter include:

- coinheritance of a 'silent' β thalassaemia allele from the mother
- coinheritance of homozygosity or heterozygosity for triple α .

The latter explanation was found to be correct; the mother and the daughter were heterozygous for triple α . This condition was harmless in the mother but in the daughter it aggravated the chain imbalance attributable to the β thalassaemia trait and led to a more severe phenotype.

Bain BJ, Swirsky D, Bhavnani M, Layton M, Parker N, Makris M *et al.* (2001) British Society for Haematology Slide Session, Annual Scientific Meeting, Bournemouth, 2000. *Clin Lab Haematol*, **23**, 265–269.

Exercise 8.22

The findings are those of heterozygosity for $\delta\beta$ thalassaemia. Hereditary persistence of fetal haemoglobin is excluded by the 'thalassaemic' red cell indices. Note that in addition to the increased haemoglobin F (shaded) shown on HPLC, there are complex early peaks (to the left), which represent post-translationally modified haemoglobin F.

Exercise 8.23

The most likely diagnosis in the mother is β thalassaemia trait and in the daughter is compound heterozygosity for haemoglobin S and β thalassaemia (note the nucleated red blood cell and, on the right of the image, one sickle cell). In fact, she had S/ β^0 thalassaemia.

Exercise 8.24

Haemoglobin S/ β^+ thalassaemia compound heterozygosity.

Exercise 8.25

The most likely diagnosis is methaemoglobinemia caused by exposure to a toxic substance found in the garden shed.

Methaemoglobin should be tested for by spectrometry or by co-oximetry. A co-oximeter is an instrument that passes monochromatic light at four wavelengths through the test sample and is thus able to quantitate carboxyhaemoglobin, methaemoglobin, oxyhaemoglobin and haemoglobin.

If treatment is needed, the correct treatment is intravenous methylene blue. This drug is contraindicated in patients with glucose-6-phosphate dehydrogenase deficiency but since the patient is identified as 'English' this deficiency is unlikely.

Wentworth P, Roy M, Wilson B, Padusenko J, Smeaton A and Burchell N (1999) Clinical pathology rounds: toxic methemoglobinemia in a 2-year-old child. *Lab Med*, **30**, 311–315.

Exercise 8.26

A variant haemoglobin is present, suggesting that the polycythaemia is the result of a high affinity haemoglobin. This was haemoglobin Kempsey and although the variant haemoglobin appears in the 'D window' of the HPLC chromatogram, its curious shape is characteristic of haemoglobin Kempsey.

Exercise 8.27

The grossly increased 'P2 fraction' has nothing to do with diabetes, although it would give a factitious result on haemoglobin A_{1c} quantification. It

should be recognised as a variant haemoglobin. It was identified by mass spectrometry as haemoglobin Hope (with thanks to Dr Barbara Wild).

Exercise 8.28

The recovery with methylene blue suggests methaemoglobinemia.

Given the father's occupation, the likely underlying cause is accidental exposure to sodium nitrite, used for curing meat.

In the family described, the sodium nitrite had been introduced into the domestic environment for use as an insecticide and had been emptied into a sugar bowl by one of the children.

Finan A, Keenan P, O'Donovan FO, Mayne P and Murphy J (1998) Methaemoglobinemia associated with sodium nitrite in three siblings. *Br Med J*, **317**, 1138–1139.

Exercise 8.29

The variant haemoglobin has electrophoretic characteristics suggestive of haemoglobin E but it is odd that the patient is European. In addition, the proportions of haemoglobin A and the variant haemoglobin appear to be similar, a very unlikely finding if this were haemoglobin E. The results of HPLC analysis exclude the possibility of haemoglobin E since there is a variant haemoglobin in the 'S window'. These are the features of haemoglobin E-Saskatoon, which does not have the genetic implications of haemoglobin E. This case shows that even with two independent methods, a provisional identification can be wrong.

Exercise 8.30

The HPLC chromatogram and haemoglobin electrophoresis show the presence of haemoglobin S at an unusually low level of 20%. Haemoglobin A₂ appears slightly elevated but its quantification can be inaccurate in the presence of haemoglobin S as post-translationally modified haemoglobin S appears in the A₂ window. There is a slight increase in haemoglobin F. There

is an abnormal fraction eluting early, which has the form expected of haemoglobin Bart's (although no haemoglobin Bart's was visible on haemoglobin electrophoresis). A haemoglobin H preparation was negative. These findings are the consequence of sickle cell trait plus the genotype of haemoglobin H disease. The reported levels of haemoglobin S in this condition vary between 10% and 25%. Haemoglobin H is present in only very trivial amounts.

Exercise 8.31

The possibility of elevation of haemoglobin A₂ for another reason (e.g. treatment of retroviral infection) should be considered. Alternatively, could the indices be atypical because of coexisting liver disease, megaloblastic anaemia (unlikely as the haemoglobin concentration is normal) or hydroxycarbamide therapy (not very likely in a young preoperative patient)?

The explanation was found, on DNA analysis, to be coexisting α and β thalassaemia trait, known to normalise the red cell indices but not the elevated haemoglobin A₂ that would be expected in β thalassaemia trait. Specifically, she had $-\alpha/\alpha\alpha$ and the β^+ thalassaemia mutation, $-29 A \rightarrow G$.

Exercise 8.32

The blood film shows hypochromia, microcytosis and poikilocytosis. The nucleated red blood cell is a micronormoblast with defective haemoglobinisation. The clinical features, in the light of the total absence of haemoglobin A, are indicative of β thalassaemia intermedia.

This was found to be due to compound heterozygosity for β^0 thalassaemia (mutation IVS2-849 $A \rightarrow G$) and the Ghanaian deletional hereditary persistence of fetal haemoglobin (HPFH-2). The phenotype of this compound heterozygous state is often milder than in this patient.

Exercise 8.33

The blood film shows target cells and irregularly contracted cells.

The infant has biochemical evidence of iron deficiency. Considering the ethnic origin, the variant haemoglobins are likely to be haemoglobin E (from her Thai mother) and haemoglobin C (from her African Caribbean father). This provisional diagnosis was confirmed by cellulose acetate electrophoresis at alkaline pH and acid agarose electrophoresis. Note that haemoglobin E (plus A₂) is only 30.4% while haemoglobin C is 57.8%, reflecting the fact that haemoglobin E is a thalassaemic haemoglobinopathy. Note also the two peaks of post-translationally modified haemoglobin E and two of post-translationally modified haemoglobin C.

Spencer-Chapman M, Kiritkumar K, Lund K and Bain BJ (2019) An unusual hemoglobinopathy: compound heterozygosity for hemoglobins C and E. *Am J Haematol*, **94**, 144.

Exercise 8.34

The HPLC chromatogram shows the double peak of haemoglobin H that is characteristic of this instrument. This finding, together with the blood count, confirms a diagnosis of haemoglobin H disease. Note the low haemoglobin A₂ percentage (1.4%) which is typical of this condition (although in this instance it is partly artefactually lowered as the baseline is high).

The most likely ethnic origin is South-East Asian (Thai, Cambodian, Laotian, Malaysian or Filipino) or Chinese.

Exercise 8.35

The patient has sickle cell trait and diabetes mellitus. The peaks from left to right are: injection artefact, haemoglobin F, glycated haemoglobin A (8.3%), other post-translationally modified haemoglobin A, haemoglobin A₀ with the shoulder on the left of the main peak representing glycated haemoglobin S, haemoglobin A₂ (plus some post-translationally modified haemoglobin S) and haemoglobin S₀.

It is important to recognise the evidence of diabetes mellitus and draw this to the attention of clinical staff if this diagnosis is not already known.

Exercise 8.36

The results of HPLC were misinterpreted in the father. A small peak in the haemoglobin S window could represent carry-over from a previous specimen but in fact, he had haemoglobin A₂' and this should have been added to haemoglobin A₂; the sum of haemoglobin A₂ and the variant of 5.5% would have revealed that he, as well as the mother, had β thalassaemia trait. The baby has β thalassaemia major.

Exercise 8.37

The noxious vapour was carbon monoxide. Carbon monoxide poisoning causes death by asphyxiation. Carboxyhaemoglobin has no

oxygen-combining activity and, in addition, increases the oxygen affinity of the remaining haemoglobin, further impairing oxygen delivery to tissues.

The servants' instinctive action in throwing open the windows has a sound physiological basis since carboxyhaemoglobin is slowly converted to oxyhaemoglobin on breathing room air. Removing Madame Zola to another room may have been even more effective.

Ashcroft J, Fraser E, Krishnamoorthy S and Westwood-Ruttledge S (2019) Carbon monoxide poisoning. *BMJ*, **365**, 409–411.

Appendix: electronic resources

Sickle cell disease (specific)

Guidelines

American Society of Hematology. Clinical Practice Guidelines on Sickle Cell Disease. www.hematology.org/education/clinicians/guidelines-and-quality-care/clinical-practice-guidelines/sickle-cell-disease-guidelines

British Society for Haematology. Management of Acute Chest Syndrome in Sickle Cell Disease, 2015. <https://b-s-h.org.uk/guidelines/guidelines/management-of-acute-chest-syndrome-in-sickle-cell-disease/>

British Society for Haematology. Red cell Transfusion in Sickle Cell Disease, 2016. <https://b-s-h.org.uk/guidelines/guidelines/red-cell-transfusion-in-sickle-cell-disease-part-1/> <https://b-s-h.org.uk/guidelines/guidelines/red-cell-transfusion-in-sickle-cell-disease-part-ii/>

British Society for Haematology. Guidelines for the use of Hydroxycarbamide in Children and Adults with Sickle cell Disease, 2018. <https://b-s-h.org.uk/guidelines/guidelines/guidelines-for-the-use-of-hydroxycarbamide-in-children-and-adults-with-sickle-cell-disease/>

National Institute for Health and Care Excellence (NICE). Sickle Cell Disease: Managing Acute Painful Episodes in Hospital. Clinical guideline CG143, 2012. www.nice.org.uk/guidance/cg143

National Institute for Health and Care Excellence (NICE). Sickle Cell Disease. Quality standard QS58, 2014. (Deals with painful crisis.) www.nice.org.uk/guidance/qs58

Public Health England. Sickle Cell Disease in Children: Standards for Clinical Care, 2010. www.gov.uk/government/publications/sickle-cell-disease-in-children-standards-for-clinical-care

Sickle Cell Society. Standards for the Clinical Care of Adults with Sickle Cell Disease in the UK, 2018. www.sicklecellsociety.org/wp-content/uploads/2018/04/Web-version-FINAL-SCS-Standards-GSM-6.4.18.pdf

WHO Model List of Essential In Vitro Diagnostics. Gives list of minimal essential tests for diagnosis of sickle cell disease. <https://edl.who-healthtechnologies.org>.

Websites

American Society of Hematology. Sickle Cell Advocacy. www.hematology.org/advocacy#:~:text=Sickle%20Cell%20Disease%20Advocacy,of%20Health%20and%20Human%20Services.

ENERCA. Clinical recommendations for disease management and prevention of complications of sickle cell disease in children. <https://online.library.wiley.com/doi/10.1002/ajh.21865>

Global Alliance of Sickle Cell Disease Organisations. www.globalscd.org

Sickle Cell Disease Association of America (patient support group). www.sicklecelldisease.org

Sickle Cell Information Centre, Atlanta, Georgia. www.sicklecellga.org/#:~:text=Sickle%20Cell%20Foundation%20of%20Georgia,Mays%20Drive%20SW%20Atlanta%20GA

Sickle Cell Society (UK patient support group). www.sicklecellsociety.org/

Sickle in Africa. www.sickleinafrica.org/

Consortium for Newborn Screening in Africa. www.hematology.org/global-initiatives/consortium-on-newborn-screening-in-africa

Haemoglobinopathy Diagnosis, Fourth Edition. Barbara J. Bain and David C. Rees.

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Université Paris Cité course on sickle cell disease. <https://odf.u-paris.fr/fr/offre-de-formation/diplome-d-universite-1/sciences-technologies-sante-STS/du-enseignement-on-line-sur-la-drepanocytose-I73G5WVE.html>

World Coalition on Sickle Cell Disease. www.worldscdcoalition.org

Thalassaemia (specific)

Guidelines

Stephens AD, Angastiniotis M, Baysal E, Chan V, Fucharoen S, Giordano PC *et al.*, International Council for the Standardisation of Haematology (ICSH) (2012) ICSH recommendations for the measurement of haemoglobin A2. *Int J Lab Hematol*, **34**, 1–13. doi: 10.1111/j.1751-553X.2011.01368.x. <https://onlinelibrary.wiley.com/doi/full/10.1111/j.1751-553X.2011.01368.x>

Stephens AD, Angastiniotis M, Baysal E, Chan V, Davis B, Fucharoen S *et al.*, International Council for the Standardisation of Haematology (ICSH) (2012) ICSH recommendations for the measurement of haemoglobin F. *Int J Lab Hematol*, **34**, 14–20. doi: 10.1111/j.1751-553X.2011.01367.x. <https://onlinelibrary.wiley.com/doi/full/10.1111/j.1751-553X.2011.01367.x>

Thalassaemia International Federation. Guidelines for the Management of α -Thalassaemia. <https://thalassaemia.org.cy/publications/tif-publications/guidelines-for-the-management-of-alpha-thalassaemia/>

Thalassaemia International Federation. Guidelines for the Management of Non-Transfusion Dependent β -Thalassaemia. <https://thalassaemia.org.cy/publications/tif-publications/guidelines-for-the-management-of-non-transfusion-dependent-beta-thalassaemia-3rd-edition-2023/>

Thalassaemia International Federation. Guidelines for the Management of Transfusion Dependent Thalassaemia. <https://thalassaemia.org.cy/publications/tif-publications/guidelines-for-the-management-of-transfusion-dependent-thalassaemia-4th-edition-2021-v2/>

Websites

Thalassaemia International Federation. <http://thalassaemia.org.cy/>

UKTS (previously UK Thalassaemia Society, patient support group). www.ukts.org/

Cooley's Anemia Foundation (patient support group). www.thalassemia.org/

Thalassaemia Society of NSW (patient support group). <https://thalnsw.org.au>

General (both sickle cell and thalassaemia, or other haemoglobinopathy)

Guidelines

Accessible Publication of Genetic Information (ApoGI), University College, London. <http://2000.apogi.info/data/html/hb/menu.htm>

British Society for Haematology. Significant Haemoglobinopathies: Guidelines for Screening and Diagnosis, 2023. <https://b-s-h.org.uk/guidelines/guidelines/significant-haemoglobinopathies-a-guideline-for-screening-and-diagnosis>

Public Health England. NHS Sickle Cell and Thalassaemia (SCT) Screening Programme, 2013 with later updates. www.gov.uk/topic/population-screening-programmes/sickle-cell-thalassaemia

Public Health England. NHS Sickle Cell and Thalassaemia Screening: Handbook for Laboratories, 2017 with later updates. www.gov.uk/government/publications/sickle-cell-and-thalassaemia-screening-handbook-for-laboratories

Sickle cell and thalassaemia screening: handbook for newborn laboratories. GOV.UK. 2022. www.gov.uk/government/publications/sct-screening-handbook-for-newborn-laboratories

Sickle cell and thalassaemia screening: handbook for antenatal laboratories. GOV.UK. 2022. www.gov.uk/government/publications/sct-screening-handbook-for-antenatal-laboratories

Population screening programmes: NHS sickle cell and thalassaemia (SCT) screening programme. GOV.UK.2023. www.gov.uk/government/collections/nhs-sickle-cell-and-thalassaemia-sct-screening-programme

Traeger-Synodinos J, Harteveld CL, Old JM, Petrou M, Galanello R, Giordano P *et al.*, on behalf of contributors to the EMQN Haemoglobinopathies Best Practice Meeting. EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies. www.ncbi.nlm.nih.gov/pmc/articles/PMC4666573/

Websites

Bio-Rad library of haemoglobin variants. <https://hemoglobins.bio-rad.com/Pages/StartPage.aspx>

Caribbean Network of Researchers in Sickle Cell Disease and Thalassaemia. <https://carest-network.org>

National Haemoglobinopathy Panel (UK). Website containing educational material and guidelines on sickle cell disease and thalassaemia. www.nationalhaempanel-nhs.net

National Haemoglobinopathy Register (UK). <https://nhr.mdsas.com>

Sebia online atlas of capillary electrophoresis. <https://extranet.sebia.com/user>

Ithanel. An international thalassaemia and haemoglobinopathy genetic information resource. <http://www.ithanel.eu/>

Public Health England. NHS Newborn Blood Spot (NBS) Screening Programme. www.gov.uk/topic/population-screening-programmes/newborn-blood-spot

Sickle Cell and Thalassaemia Association of Nurses, Midwives and Allied Professionals. <https://stanmap.org.uk>

South Thames Sickle Cell and Thalassaemia Network. <http://www.ststn.co.uk>

The Globin Gene Server, hosted by Pennsylvania State University, USA and McMaster University, Canada. <http://globin.cse.psu.edu/>

Thalassaemia and Sickle Cell Australia (patient support group). www.tasca.org.au/

UK Forum for Haemoglobin Disorders (a multidisciplinary group of healthcare professionals involved in thalassaemia and sickle cell disease care). www.haemoglobin.org.uk

Genes

Accessible Publication of Genetic Information (ApoGI), University College, London. <http://2000.apogi.info/data/html/hb/menu.htm>

Human Genome Variation Society nomenclature for the description of DNA, RNA and protein sequence variants. <https://hgvs-nomenclature.org>

Ithanel (resource provided by an international co-operative group giving genetic information on haemoglobinopathies). <http://www.ithanel.eu/db/ithagenes>

Globin Gene Server. <http://globin.cse.psu.edu/>

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